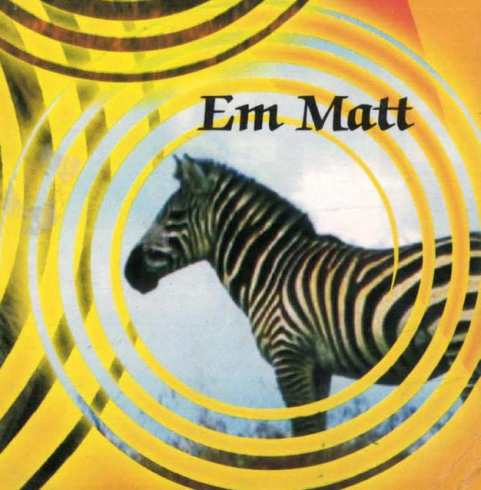
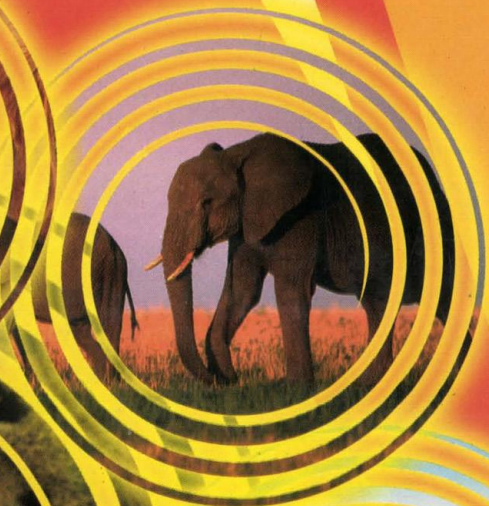


# Your Guide to **ANIMAL** Biotechnology



**Em Matt**

# **Your Guide to Animal Bio-Technology**

**Edited by  
Em Matt**



**ABHISHEK PUBLICATIONS  
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# Preface

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Animal Biotechnology is not a modern area of study. The application of different biological technologies to animals started with ice age in the South West Asia, when humans first began to trap wild animal species and tried to breed them in captivity, initially for meat and fibre and later for transport and milk. For different reasons, the scientists are concerned about the outcome of the different technologies that have been engineered with the animals. The area of Animal Biotechnology is very vast. This area of study includes subjects like human genes; its most significant example being adding human genes to sheep so that they secrete alpha-1 antitypes in their milk, which is useful in treating some cases of lung diseases. Using this technology, bacteria was produced with human genes for insulin in 1983, which proved to be a great practical application that saved many lives. The technology has also been applied to produce a chicken with four legs and no wings. Scientists have created a goat with spider genes that creates silk in its milk.

Biotechnology allows us to combine fish, mouse, human and insect genes in the same person or animals. Genetic based animal biotechnology has produced new food and pharmaceutical products and promises many more advances to benefit human kind. However, these exciting prospects are accompanied by considerable uneasiness about matters like safety and ethics.

Animal disease is a major area of concern and it can threaten the



globe. Biotechnology has sincerely attempted to find out some major animal diseases and suggested their control methods. The use of drugs in animal food production has resulted in benefits throughout the food industry; however, their use has also raised public health and safety concerns.

This book gives an overview of why and how drugs are used in the major food producing and animal industries, like poultry, dairy, beef and aquaculture. The prevalence of human pathogens in foods of animal origin has also been discussed. Focus has been directed on areas like the transfer of resistance in microbes to human pathogens and the resulting risk of human diseases.

This study of animal biotechnology includes a short description of all the concerned areas. It provides the definition of major areas like cloning, nuclear transfer, cell culture, etc. Looking at technologies on the near horizon, topics like what the scientists know and what they fear about, the inadvertent release of dangerous micro-organisms, the safety of products derived from genetic animals, the impact of genetically engineered animals on their environment, etc. are also discussed.

This book is a must read for everyone interested in the implications of the use of animal biotechnology.

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## The Application of Biotechnology to Animals

Animal biotechnology is not a new area of study. The application of different biological technologies to animals started with ice age in the Southwest Asia when humans first began to trap wild animal species and tried to breed them in captivity, initially for meat and fibre and later for transport and milk. Of the approximately 48,000 mammalian species, fewer than 20 have been successfully domesticated. Other than cats and dogs, only five of these species (cattle of the *Bos* genus, whose ancient ancestor is the now extinct auroch, sheep derived from the Asiatic mouflon species, goats, which are descended from the bezoar goat of West Asia, pigs derived from captured wild boars; and horses, which originated from now extinct wild horses that roamed the steppes of Southern Russia) are found worldwide.

The animals that have been successfully domesticated and farmed share and exhibit a unique combination of characteristics. They are relatively docile, are flexible in their dietary habits and can grow and reach maturity quickly on a herbivorous diet and breed readily in captivity. They also have hierarchical social structures that permit humans to establish dominance over them and are adapted to living in large groups. They do not include species that generally have a tendency to be fearful of humans or disturbed by sudden changes in the environment. Our ancestors no doubt based their selection methods for improving their herds and flocks on how easy the animals were to

farm, as well as on potential agricultural value. In turn, the animals are adapted to thrive in a domesticated environment.

## **Animal Biotechnology — Area of Concerns**

It is observed that animal biotechnology has not been treated as a safest area of study. For different reasons the scientists are concerned about the outcome and utility of the technology. The first is whether anything theoretically could go wrong with any of the technologies. For example, is it theoretically possible that a vector used for gene transfer could escape and become integrated into the DNA of another organism and thereby create a hazard? The second is whether the food and other products of animal biotechnology, whether genetically engineered or not or from clones, are substantially different from those derived by more traditional, extant technologies. A third major concern was whether the technologies raise novel environmental issues and a fourth was whether they raise animal welfare issues.

Finally, there was concern as to whether the statutory tools of the various government departments and agencies involved are sufficiently well-defined and whether the technologic expertise and capacity within agencies are sufficient to cope with the new technologies, should they be deemed to pose a hazard.

## **Recent traits in Animal Biotechnology**

Today we feel a severe pressure on the demand of meat. Infact our agricultural lands are deteriorating day by day. As a result there is pressure to utilise the potential for biotechnology to improve productivity in animal agriculture. As the techniques for producing transgenic animals become more efficient and as more is known about controlling how inserted genes are expressed, it is likely that the approaches soon can be integrated into agriculture. Indeed, the commercial production of transgenic fish, which is likely to occur worldwide, already is imminent.

Genetically engineered poultry, swine, goats, cattle and other livestock

also are beginning to be used as generators of pharmaceutical and other products. The technology to produce foreign proteins in milk by expressing novel genes in the mammary glands of livestock already has advanced beyond the experimental stage, with some of the products currently in clinical trials. In theory, transgenic animals can provide milk that is more nutritious for the consumer or that is enhanced for certain protein components that might be valuable for manufacturing cheese or other dairy products. However, the largest investments in the technology to date have been made by pharmaceutical companies interested in producing enzymes, clotting factors and other bioactive proteins in milk.

Today different companies are interested in farm animals because they are considered as a strong source of replacement organs for humans. Transplantation is an accepted and successful treatment for organ failure, but there is an enormous shortage of available human organs. As there are ethical and practical concerns related to the use of donor organs from primates, the pig, in particular, is being considered as an alternative. Unfortunately, humans express antibodies to a carbohydrate epitope that is present on the surface of pig cells. As a result, the xenograft immediately becomes a target for acute rejection. To remedy this situation, pigs will be produced that lack the 1,3 galactosyl transferase enzyme.

Because of its small size, short generation times, fecundity and well-studied genetics the mouse has become the animal of choice for providing models for human disease. Farm species might provide alternatives where the mouse is inappropriate. One possible future scenario is the creation of specific gene knockouts in farm animals in order to mimic human disease in a large animal model. For example, McCreath, have generated genetically engineered sheep carrying a mutated collagen gene and have suggested that such animals could serve as models for the human connective tissue disease

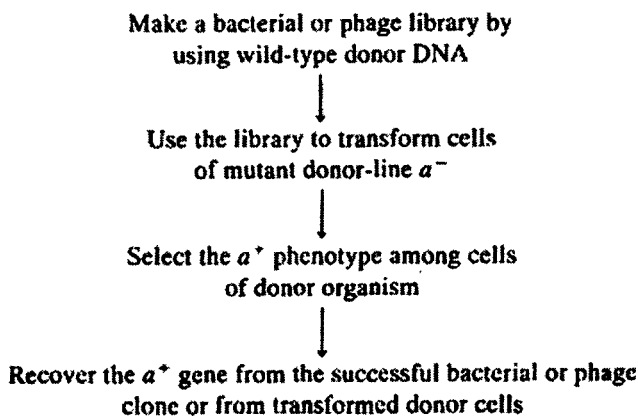
These new technologies and their practice raises concerns about whether the end products can be consumed safely, whether there are likely to be unwanted effects on the environment and whether animal



welfare will be adversely affected. However, before proceeding further, it is perhaps helpful to understand what is meant by biotechnology and to appreciate how far such biotechnology already has been incorporated into current agricultural and biomedical practice. It also is clear that the concerns of the public are focused on some of the more recent technologic advances relating to gene transfer between organisms that would not normally interbreed and to assisted reproductive procedures, such as somatic nuclear cell transfer to create so-called clones, any of these recent advances have not yet left the experimental stage, but it is clear that several, including transgenic finfish, which are soon likely to be commercialised, are likely to assume importance both in agriculture and medicine.

## The meaning of Cloning

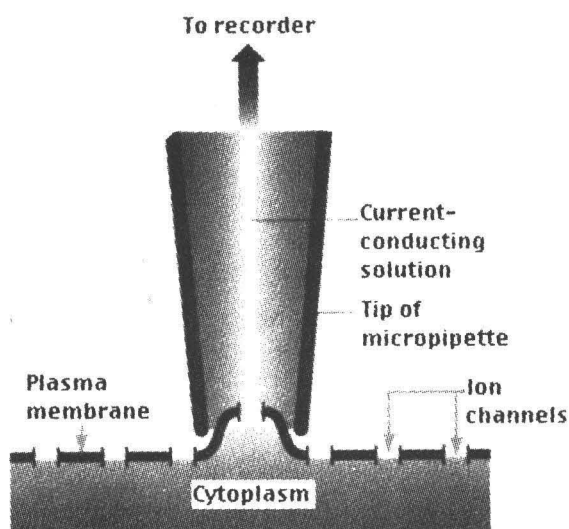
‘To clone’, means to make a copy of an individual. ‘Clone’ was adopted into the parlance of modern cellular and molecular biology to describe groups of



identical cells and replicas of DNA and other molecules. Monozygotic twins are clones, but the term has recently become popular in the media to mean an individual, usually a fictitious human, grown from a single somatic cell of its parent. The first reports of animal cloning were reported in the late 1980s, which were the result of the transfer to anucleated oocytes of nuclei from blastomeres (cells from early and presumably undifferentiated, cleavage-stage embryos), a technique that is referred to as blastomere nuclear transfer or BNT. Cloning of sheep,

cattle, goats, pigs, mice and, more recently, rabbits and cats, by transplanting a nucleus from a somatic and presumably differentiated, cell into an oocyte—from which its own genetic material has first been removed—was achieved about a decade later, leading to the speculation that humans also could be cloned. It is important to note that somatic cell nuclear transfer (SNT) also can be used to produce embryonic stem cells, giving researchers the opportunity to obtain undifferentiated stem cells that are genetically matched to the recipient for research and therapy, which is independent of the discussion here regarding the use of SNT for reproductive cloning of animals. Neither BNT nor SNT result in an exact replica of an individual animal, although the progeny are very similar to each other and to their donor cell parent. Any genetic dissimilarity is likely due to the cytoplasmic

inheritance of mitochondria from the donor egg, which possesses its own DNA and to other cytoplasmic factors, which seem to have the potential to influence the subsequent 'reprogramming' of the transferred somatic cell genome in such a way that spatial and temporal patterns of



gene expression in the embryo are affected as it develops. For these reasons, many scientists have objected to the use of the term clone in the context of somatic cell nuclear transfer. Nevertheless, clone is now so widely accepted as a synonym for somatic cell nuclear transfer—not just by the public at large—but also by embryologists and other biologists.

## Different Technologies Associated with Modern Animal Agriculture

Vaccinations and other health technologies; Vaccination is used widely in the livestock and poultry industries as a protection against viral and bacterial pathogens.

Artificial insemination; Artificial insemination (AI)—in conjunction with the use of frozen semen from select bulls— is common in the dairy industry but relatively rare in the U.S. beef industry. The use of fresh semen for AI is becoming increasingly important in the swine and poultry industries. Artificial insemination, which was first described in 1910 but not widely adopted until the 1950s.

- Freezing of semen
- Sire testing and selection
- Use of antibiotics in feed to increase gain
- Embryo transfer
- Embryo splitting and cloning from blastomeres
- In vitro maturation/in vitro fertilisation of oocytes and in vitro culture of resulting embryos
- Use of hormones to control ovulation in farm animals and to induce spawning in fish zygotes
- Hormonal sex reversal and production of monosex fish stocks
- Chromosome set manipulation
- Steroid administration to improve weight gain
- Bovine somatotropin (BST) to increase milk production in dairy and swine industries.



With the use of these technologies we can develop different breeds of new species. But they differ so markedly from their progenitor species. It shows that how quickly directed breeding can act. The modern Holstein, which dominates the contemporary United States dairy industry, little resembles its ancestors of only a half-century ago. Milk production per cow increased almost threefold between 1945 and 1995, largely as a result of breeding from select bulls. There has been an accompanying drop in the number of cows, land devoted to dairy production and in manure produced. On the downside, the cows have a tendency towards lameness, are considerably less fertile than in the 1940s and are frequently maintained in a herd for no more than 2–3 years or 2–3 lactations and represent a very narrow genetic lineage. The export of these animals and their lineages to Europe and elsewhere is assuring the globalisation of both the benefits and drawbacks of the American Holstein. Analogous changes are ongoing in the swine industry, where the pressure to produce lean, fast-growing animals of uniform size is leading to the abandonment of old breeds. Paradoxically, unless other hand provides the old livestock breeds are eaten, sheared or milked, they will not survive.

The study of dog (*Canis familiaris*), on the interesting example of the range of phenotypes that can be derived by selection within a single species. Dogs are believed to have originated in several separate domestications from wolves (*Canis lupus* and *Canis rufus*) and coyotes (*Canis latrans*) before the domestication of livestock. They have undergone remarkable modifications in size and behaviour over short periods of intense selection and to provide the diversity observed in modern breeds. This reflects the enormous pool of genetic variation within the species, but (possibly) also the fixation of new mutations into different genetic lineages. Inbreeding of dog breeds, as of domestic livestock, has led to a major narrowing of intrabreed variability.

Different modern breeds of farmed fowl include chickens, ducks, geese and turkeys, which are domesticated for their meat, eggs and feathers. As in the dairy industry, there has been a remarkable improvement in the productivity of the poultry industry over the last 60 years. Between 1940 and 1994, yearly egg production per laying hen increased from

134 to 254, mainly as a result genetic selection. The broiler industry has shown similar gains. In 1950, a commercial bird took 84 days to reach a market weight of 1.8 kilogram. By 1988, this market weight was reached by only 43 days on about half the amount of feed.

Now we have produced different new breeds of animals, which are remarkably productive, and sometimes strikingly different in habits and appearance from those farmed early in the twentieth century. This is the result of scientific breeding, better nutrition and veterinary care. The practice has also led to a loss of many breeds of livestock and fowl and a decline in genetic diversity within the breeds that survive. For example, it has been estimated that there were several hundred-specialty lines of chicken in North America at the beginning of the last century, whereas the number of commercial hybrid strains now available through suppliers is fewer than 10.

In a major setback different aquatic animals, including finfish and shellfish, now are farmed and specific breeds that have been selected for growth and other traits are established now in the largest industrial sectors of aquaculture, such as channel catfish, rainbow trout and Atlantic salmon. The growth and quality of such animals are also amenable to genetic engineering through modern biotechnology. Genetically engineered or highly selected aquatic species present special problems in terms of confinement, as the features that might make them attractive commercially might pose risks to the genetic base of their wild relatives with which they can interbreed.

Domesticating insects for farming is a novel idea. The two best-known examples are the honeybee and silkworm. Considerable genetic gains in productivity have provided strains of these insects far removed from the ancestral species from which they were derived. Attempts to develop strains of honeybee with improved resistance to pathogens and silkworms that produce proteins other than silk are on the horizon. In addition, insects, including ones that can be engineered transgenically, are likely to continue to be used as part of biocontrol programs for pest insects and invasive plant species and, as such, might be intentionally released into the environment. There will almost certainly be attempts to replace or to infiltrate native populations

with insects that have been engineered in such a manner that they are less of a pest or unable to transmit pathogens. Private-sector companies already have begun to farm recombinant proteins (antibodies, cytokines, enzymes and bioactive peptides) from insect larvae. Horizontal gene transfer, disruption of ecosystems and native species extinctions are among the potential hazards that arise from permanent releases of transgenic arthropods into the environment.

## **Experimentally Established Technologies in Animal Agriculture**

1. Production of transgenic animals through genetic engineering of sperm
2. Cloning of adult animals by somatic cell
3. Cloning of animals by somatic cell nuclear transfer to achieve genetic engineering
4. Nuclear transfer to produce 'copies'
5. Production of sexed semen
6. Production of transgenic animals by direct gene transfer.

Importantly, other forms of research-driven biotechnologies, based on improved insight into reproductive physiology and endocrinology, embryology, genetics and animal health also have made their way into standard farming practices over the last 75 years. A few of the procedures listed extend the boundaries of biotechnology to the development of organisms that have a combination of traits generally not attainable in nature through conventional breeding and are not themselves without controversy. Finally, some of the concerns raised about the technologies are quite relevant. Several of these technologies remain experimental and have not yet become a part of standard agricultural practice; others (e.g., commercialisation of transgenic fish) are undergoing government review for commercial approval. For these reasons, it is worthwhile discussing some of the issues that these technologies have raised.



## Expressed concerns regarding technologies

### Subtherapeutic Use of Antibiotics

In 1951 antibiotics were approved as feed additives for animals by Food and Drug Administration. Their use since has been extended to fish farming,

particularly with the global spread and dramatic increase of aquaculture in tanks and pond-

like structures where antibiotics are used for prevention and control of disease rather than to enhance growth. The treated animals are found to grow more quickly and utilise feed more



efficiently than animals on regular feed. At least 19 million pounds of antibiotics are used annually for subtherapeutic purposes in animal agriculture and generally are added to feed and water. Some of these compounds, used on livestock, including penicillin, tetracycline and fluoroquinolone used on livestock, also are prescribed to treat human illnesses and the practice has been shown in a few instances to contribute to antibiotic resistance of human pathogens. It now is generally accepted in the scientific and medical communities that antibiotic resistance can be exacerbated by the widespread improper use of antibiotics. What remains controversial is whether agriculture contributes sufficiently to the problems associated with resistant pathogens to justify a complete curtailment of their use as growth promoters. A recent report from the National Research Council failed to find a definitive link between the agricultural use of antibiotics in animal feed, drinking water and antibiotic resistance of human pathogens. The report states, 'The use of drugs in the food production

industry is not without some problems and concerns, but does not appear to constitute an immediate public health concern.' Since that report was released, additional information, raising further concerns, has been released.

## **Maintaining Animal Health**

For maintaining animal health we have established guidelines for the application of different technologies like standard vaccination against viral and bacterial diseases. Indeed considerable efforts are being made to expand the range of such technologies in order to prevent epidemic spread of disease in flocks and herds, which are particularly at risk when farmed under intense conditions. Even the therapeutic use of antibiotics to treat animals that have bacterial infections or are in danger of becoming infected seems not in itself to be controversial, except when antibiotics of medical importance to humans are employed.

## **Different Procedures of Reproduction**

There is an attempt to improve the genetic quality of farmed species. For this, different methods like, artificial insemination (AI) associated use of frozen semen, and sire testing are taken. AI, when first introduced into agriculture, elicited an enormous outcry from farmers and religious groups. It was claimed to be against the laws of God, a repugnant practice that would lead to abnormal outcomes and economically unsound. It gradually has become an accepted practice in agriculture, as well as in human and veterinary medicine. The ability to freeze semen and maintain a high degree of fertilising ability after thawing extended the power of AI, since a few select bulls could be utilised to inseminate many females in different geographic areas. Such bulls could be tested, not only for fertility, but also for their ability to sire progeny that produced copious amounts of milk. By maintaining accurate records, breeding value estimations of particular bulls could be calculated. The result was the remarkable increase in milk production, noted earlier. On the other hand, the process is leading to potentially destructive inbreeding since many of the select bulls are related.

Inbreeding coefficients among modern Holsteins and Jersey breeds are now about 5 percent and rising. The outcome might be inbreeding depression and broad susceptibility to the epidemic spread of disease. There also has been a remarkable recent loss of fertility, with successful pregnancies resulting from first insemination dropping from more than 40 percent to as low as 20 percent or less in some herds as milk yields have risen.

It is now possible for an animal to parent many more offspring in her lifetime through the process of embryo recovery. The embryos also can be frozen and then either stored or transported before they are used to initiate a pregnancy. It is a relatively common technology and has been used to produce an estimated 40,000 to 50,000 thousand beef calf every year. The approach is to induce, by using hormones, the maturation and release of more than a single egg from the ovaries. Then, the animal usually is inseminated with semen from an equally select bull and the embryos are collected and transferred individually or in pairs, to the reproductive tract of less valuable cows, which carry the calf to term. Modern technologies also provide the possibility of freezing the embryos and determining their gender prior to transfer. The main concern with this technique, as with the AI-associated technologies discussed above, is that it can lead to narrowing of the genetic base of the breed, in this case involving both parents. A related technique is to use a needle to aspirate immature oocytes from the ovaries (in the case of livestock the oocytes often are taken from slaughtered animals at an abattoir) and to mature the oocytes for about one day in a culture containing hormones. At the stage when the oocytes reach a point midway through the second division of meiosis, they are fertilised with live sperm. In rare instances, fertilisation is achieved by a single sperm or sperm head, which is injected through the tough outer zona pellucida of the oocyte, either beneath the zona or directly into the cytoplasm (intracytoplasmic injection or ICSI). Whatever method is used for fertilisation, the resulting zygotes usually are then cultured until the embryo reaches a more advanced stage of development. In humans, of course, these combined techniques form the basis of in vitro fertilisation procedures and have resulted in hundreds of thousands of normal infants, but the techniques also

have become an important means of producing embryos for experimental purposes in agricultural research. Importantly, in vitro maturation of oocytes underpins cloning and transgenic technologies, where large numbers of competent, matured oocytes are needed to provide the many eggs necessary for nuclear transfer and pronuclear injection, respectively. In vitro fertilisation also is used commercially to preserve the genome of particularly valuable animals that have infertility problems such as blocked oviducts or that respond poorly to superovulation, a technique described below. This commercial application of IVF is a relatively uncommon, with about 4,000 calves born from its use annually. Few concerns have been raised about this technique, which essentially is identical to that employed for in vitro fertilisation in humans, although some animal welfare issues have been raised. Control over the reproductive cycles of livestock by hormonal intervention has increased in order to manage breeding programs more intensively.

In general the technologies are relatively benign and involve injecting the animal with hormones, usually to stop progression through the existing estrous cycle and sometimes to mimic the events that lead to selection of one or more mature follicle(s) that will ovulate. Superovulation is a technique designed to mature a cohort of follicles simultaneously, with result that several eggs are ovulated simultaneously. A hormone treatment analogous to that used to produce a timed ovulation in the large farm animals is used to induce gonadal maturation in fish. None of these techniques have raised public health concerns, since the hormones are similar or identical to those in normal reproduction and the amounts used within the physiologic range.

In order to provide zygotic twins splitting or bisecting embryos became an esoteric but well-established practice in 1980. The pieces of the embryo—usually ‘halves,’ which are genetically identical in terms of both their nuclear and mitochondrial genes—are placed in an empty zona (the protective coat around early embryos) before being transferred to different recipient mothers to carry them to term. It is estimated that only a very small number of the calves (1 to 2 percent

of those resulting from embryo transfer in the United States and Canada) are produced in this manner. Nevertheless, these animals have been introduced into commercial herds and have produced progeny; their milk and meat are consumed by the public.

In 1970, a new method of cloning was done. It was done by nuclear transplanting from embryonic blastomers. It is a very expensive procedure. What distinguishes it from somatic cell nuclear transfer, the technology that led to the creation of Dolly and much of the controversy over human cloning, is the stage of development at which the nuclei are transferred. In the older procedure, the cells or blastomeres used were from the so-called morula stage of cell development (although some were from the cleavage stage and others from the blastocyst stage) when the embryo still is an undifferentiated mass and its cells presumed still capable of forming all tissues of a foetus.

Embryonic nuclear transfer and embryo splitting technologies found it's way to cattle breeding in the 1980s. The Animal Improvement Programs Laboratory of the USDA's Agricultural Research Service (ARS) is responsible for tracking the performance of dairy cattle throughout the U.S. Recently, working with the Holstein Association, they evaluated the performance of cloned Holsteins produced by EMS and NT. The numbers of EMS and NT clones were documented by gender and birth year. All NTs were from embryos rather than adult cells. Through 2001, there were a total of 2,226 EMS (754 males and 1,472 females) and 187 NT (61 males and 126 females) Holstein clones registered. Of female EMS clones, 921 had yield records and 551 had noncloned full siblings with yield records. Of the 126 female NT clones, 74 had yield records, but only 11 had noncloned full siblings. These familial relationships were used to compare the performance of cloned and noncloned full siblings for standardised traits and genetic evaluations as part of the national evaluation program. These standardised traits included total milk yield, fat content (by weight and percent), protein content (by weight and percent), somatic cell score and productive life (in months). Also calculated were yield from contemporaries and predicted transmitting ability. The numbers

of clones have decreased for EMS males and for all NT clones over the past decade. Animals that were selected for cloning were slightly superior genetically to the contemporary population mean for yield traits, the yields of NT clones were similar to and those of EMS clones were slightly less than those of their noncloned full siblings.

The modern method of cloning involves taking an unfertilised egg, removing its chromosomes and introducing the nucleus from a differentiated cell of the animal to be cloned, which is frequently an adult. The introduced nucleus is reprogrammed by the cytoplasm of the egg and directs the development of a new embryo, which is then transferred to a recipient mother to allow it to develop to term. The offspring formed will be identical to their siblings and to the original donor animal in terms of their nuclear DNA, but will differ in their mitochondrial genes and possibly also in the manner their nuclear genes are expressed or biochemically engineered. Cloning from blastomeres, the older of the two procedures, has been reported to result occasionally in large calves (and lambs), the so-called large offspring syndrome. Analogous, though possibly more serious, abnormalities might be associated with cloning from somatic cells and are discussed further in chapters of this report.

## **Treatment of Hormones**

The use of steroid hormones to increase weight and to reduce accumulation of fat of young heifers is considered as a most dependable technology. The steroids are administered by slow release from a plastic implant embedded beneath the skin of the ear, which provides 'physiologic' circulating levels of the hormone in the bloodstream. The hormones used are mainly Zeranol, a naturally occurring fungal metabolite (zearalenone) with estrogenic action, estradiol, progesterone and testosterone or mixtures of these steroids and trenbolone. Concern about these hormones is probably, in part, a legacy of diethylstilbestrol, which was eventually banned from use in the poultry and beef industry because of its adverse effects on humans. However, the amounts of present-use compounds consumed from meat derived from treated cattle are small and numerous scientific



studies generally have indicated that these residues exist at such low concentrations that they pose little risk to consumers, provided good veterinary practices are employed (e.g., using the correct number of implants and placing implants correctly in the ear cartilage), although the U.S. Geological Survey has recently documented the presence of hormones in a number of streams and rivers. Despite the scientific evidence for safety, the European Union implemented a ban on U.S. beef imports, valued at over \$100 million per year in 1989. A concern that has not been extensively examined so far is whether these hormones pose any sort of environmental threat through their leaching into soil and water. For example, two recent studies have shown that a commonly used androgenic growth promotor—trenbolone—has been found in groundwater near cattle feedlots and that this growth promotor has androgenic effects.

### **The Use of BST (Bovine Somatotropin)**

This method has created many trade disputes. Bovine somatotropin has been used to increase milk yielding from dairy cows. Currently banned in Europe even for experimental studies, BST was approved by the FDA for use in U.S. dairy cattle in 1993 because testing had revealed no concerns regarding consumer safety. The Monsanto product, Posilac, now is widely used throughout the U.S. dairy industry, where milk production can be increased as much as 30 percent in well managed, appropriately fed herds, without adversely affecting the quality or composition of the milk. The BST, which is almost indistinguishable in sequence from the natural hormone, is present in low concentrations in milk, but has no biologic activity in humans. The level of IGF-1, the hormone induced by BST, is somewhat elevated but within the 'physiologic range' for cows and is probably digested along with other milk proteins in the adult stomach, although it might have biologic activity in the intestine of neonates. In its assessment, the FDA did not report that BST or IGF-1 pose any risk either in humans or animals that consume cows' milk. As with other technologies that increase productivity, a concern frequently raised is why more milk is needed when the developed world appears to have

more than enough of the product. One answer is that increased productivity translates into fewer animals, producing less waste and utilising less land—an extremely important consideration for future land management use. The greatest concerns about BST are probably in the area of animal welfare. High-yield milking cows show a greater incidence of mastitis than lower-producing cows, but studies have shown that mastitis is not exacerbated by BST administration. Another practical concern for the dairy industry is a recent trend to breed heifers only once and then to sustain milk production for as long as 600 days by using BST. Lengthening lactation via BST in second calf and older cows is a larger contributor to having fewer calves per lifetime in the herd than first-calf heifers. The result has been a shortage of replacement heifers for producers, since only one calf is born during the milking life of the animal.

## **Selection of Genetic Marker**

Marker-assisted selection involves establishing the linkage between the inheritance of a particular trait—which might be desirable, as in the case of milk yield—or undesirable, as in susceptibility to a disease, with the segregation of particular genetic markers. Thus, even if the gene that controls the trait is unknown, its presence can be inferred from the presence of the marker that segregates with it. This technology, which is particularly important for studying complex traits governed by many genes, has only recently become a factor in animal breeding and selection strategies. Its use likely will increase exponentially as the industry incorporates the data from the various genome sequencing projects and as the density of useful, segregating markers increases on the chromosomes of the species. Initially, animals will be screened for genes that control simple traits, such as horns, which are undesirable in cattle and halothane sensitivity, which segregates with metabolic stress syndrome in pigs. With time, easily identifiable markers will be chosen that accompany the many genes controlling more complex traits such as meat tenderness and taste, growth, calf size and disease resistance. The approach has enormous potential for improving the quality of agricultural products, disease resistance and other traits but

could be misused. For example, stringent selection of prime animals could potentially narrow genetic diversity even more than is evident at present. Use of the technique also could maximise short-term gain in productivity but at the expense of longer-term improvement due to what has been termed polygenic drag. In essence, the cumulative effect of genes with effects too small to be exploited in a marker-assisted selection program could contribute more to increasing desired traits than genes with major effects. However, marker-assisted selection might be a powerful measure to counter inbreeding by providing genetic measures of heterozygosity, encouraging breeding strategies that maintain diversity at the majority of sites in the genome and allowing the genetic potential of rare breeds and wild ancestors to be utilised and incorporated into mainstream agriculture.

## **Manipulation of Chromosome in Mollusks and Fish**

The alteration of the chromosome complement of animals has been proved to be a useful way to provide fertility in fish and mollusks. This technology is now exploited widely. Well-timed application of high or low temperatures, certain chemicals or high hydrostatic pressure to newly-fertilised groups of eggs can interfere with extrusion of the second polar body (the last step in meiosis), resulting in 'triploid' individuals with three, instead of the usual two, chromosome sets. A later treatment can suppress the first cell division of the zygote, resulting in 'tetraploid' individuals with four sets of chromosomes. Crossing metalloids, which are fertile in some species, with normal diploids can then produce large numbers of triploids. Such chromosome set manipulations have been applied to cultured marine mollusks to produce confined stocks of triploids that are unable to reproduce. This application is of particular importance, as some of the shellfishes most suited to aquaculture are not indigenous to a given area and can pose ecologic risks to native species should they or their larvae escape confinement and enter natural ecosystems. Induction of triploidy reduces the likelihood that an introduced species would establish self-sustaining populations, because such animals are theoretically sterile.

For example, the triploid Suminoe oyster (*Crassostrea ariakensis*) is being assessed for oyster production in the Chesapeake Bay, where diseases complicate restoration of the native Eastern oyster (*C. virginica*). Should triploidy prove an effective means for reproductive confinement, culture of sterile Suminoe oysters could support the recovery of the declining Chesapeake oyster production industry.

Another benefit of producing sterile mollusks is in maintaining product quality throughout the year. The meat quality of oysters is high just before they spawn, but low after spawning. The product quality of reproductively sterile, triploid oysters remains high year-round. Hence, triploid stocks of Pacific oyster (*Crassostrea gigas*) provide a tangible benefit to aquaculturists and now make up almost half of commercial production in the Pacific Northwest. Unfortunately, repeatable induction of 100 percent triploidy on a commercial scale poses a considerable technical challenge. Non-triploid larvae within batches of larvae easily can go undetected if their frequency is low. Should triploidy be desired for purposes of maintaining product quality and the species is indigenous to an area, no harm is posed. If, on the other hand, triploidy is to be utilised for reproductive confinement purposes, the presence of reproductively fertile individuals—even in low numbers—might establish progeny and a self-sustaining population. There also are indications that a small percentage of triploid oysters can progress to a ‘mosaic’ state, with diploid cells arising within the background of triploid cells, leading to the possibility that they could produce viable gametes.

Triploidy often has been used to reduce the likelihood that introduced finfish species. That would establish self-sustaining populations. Use of all-female triploid stocks has been suggested as a means of achieving reproductive confinement of transgenic fishes, including Atlantic salmon (the leading candidate for commercialisation). As with mollusks, however, repeatable induction of 100 percent triploidy poses a considerable technical challenge and commercial net pen operations produce hundreds of thousands of salmon, with many escaping. Another technology used on finfish is to farm monosex fish stocks, which are preferred by producers either because one gender grows

faster or larger than the other (e.g., males in catfish and tilapia, females in rainbow trout) or because certain species (e.g., tilapia) attain sexual maturity before reaching harvest size. Monosex populations have been established in several ways, but most reliably through hormone-induced gender reversal. All-male fry can be produced by direct administration of testosterone in feed or all-females by administration of estrogens. Monosex stocks also can be produced indirectly by gender reversal and progeny testing to identify XX for producing all-female stocks, as in trout and salmon or YY for producing all-male stocks, as in tilapia.

## **Related Risks and Hazards with the Technologies**

If look into the examples we will find that biotechnology has already been integrated into agricultural practices earlier. The introduction of new technologies does not mean that there are no concerns or even dangers posed by their use or that there is universal acceptance among the public. The experience of the last 50 years, if nothing else, illustrates that there must be continued vigilance even after technologies have been approved. Conversely, it should be recognised plainly that increases in agricultural efficiency brought about by new technologies, such as those discussed above, undoubtedly have contributed to a more abundant, cheaper, more varied and lower cost food supply and to enormous savings in agricultural land use. Some technologies bridge the gap between commercial practice and what is new. For example, cloning from blastomeres in reality is little different from nuclear transfer from somatic cells, listed in, except that the transferred nuclei might not have to be so extensively reprogrammed in the cytoplasm of the recipient oocyte. Similarly, chromosomal set manipulation remains partly experimental and partly an active commercial technology.

The production of single sex sperm is achieved through a cell sorting procedure that depends upon the higher DNA content of female sperm. The technology is not expected to raise any new concerns and, provided the procedure can be scaled up, is likely to be highly beneficial

in the dairy industry, where there is a surfeit of low-value bull calves and to the beef industry, where males have a higher production value than females. The remaining technologies, however, might be more worrisome to the public and to the regulatory agencies and it is these that are addressed in this report.

Some of the technologies (e.g., commercial production of transgenic finfish) already are beyond the experimental stages of development. In addition, some biopharmed drugs are in Stage 3 clinical trials and decisions must be made soon about the disposition of the livestock involved. Potential impact of the technology is an area of concern. Some new procedures seem unlikely to raise concern (e.g., the sperm sexing) or represent relatively minor changes in practice. Other technologies might be broadly adopted, yet the possible harm they could cause and the overall benefits to society are difficult to evaluate.

It is very important that we should get all the information about the technology to evaluate all the concerns properly. Unresolved scientific uncertainty interferes, not only with attempts to determine how best to apply emerging technologies to animals, but also how to predict the impacts of their application. Some hazards remain theoretical, uninvestigated, poorly characterised or even unknown. Such uncertainties present significant challenges to scientists and policy makers who wish to estimate the likelihood and distribution of harms and benefits resulting from application of those technologies. For example, some outcomes of applications of the technologies listed in, such as production of transgenic animals by gene transfer, are very difficult to predict. Uncertainties range from mere inexactness and unreliability to those that are fundamentally unknowable a priori. Clearly, technologies that pose high stakes and high uncertainties pose fundamentally different challenges than those posing low stakes and little uncertainty. Two examples of categories include companion animals and wildlife. While there are likely to be unique concerns that emerge with both categories, the concerns identified in the report regarding applications of the technologies, environmental issues and animal welfare issues are all relevant and should be included in any considerations of wildlife and companion animal species.



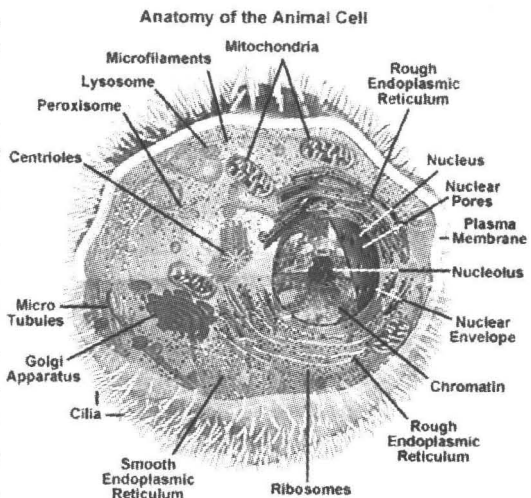
In order to provide criteria for selection of risk issues, it is important to understand how risk is determined. As outlined in and as set forth by NRC a hazard: is an act or phenomenon that has the potential to produce harm and risk is the likelihood of harm resulting from exposure to the hazard. This committee used the NRC (1996) definition of risk to develop a set of working steps to prioritise concerns. Because risk is the product of two probabilities: the probability of exposure and the conditional probability of harm given exposure has occurred, the steps in risk analysis are to: (1) identify the potential harms, (2) identify the potential hazards that might produce those harms, (3) define what exposure means and the likelihood of exposure and 4) quantify the likelihood of harm given that exposure has occurred. (The committee notes that risk analysis in other fields can and does include additional steps in risk assessment). Multiplying the resulting probabilities then was used to prioritise risk. While absolute probabilities are difficult to determine at this time, relative rankings from high to low are possible based on available evidence for each category. The risks, harms and hazards are different for each chapter because the issues are different (i.e., a hazard resulting in an animal well being concern might not be an environmental or human health concern).

GE mice are not part of the animal production system for human food and laboratory mice are highly unlikely to escape the confines of animal facilities because of their economic value and the generally high-quality care given to laboratory rodents. While mice might be a high risk for escape, might feralise easily and might carry many different transgenes, the functionality of the transgenes used in mice rarely has been for a construct that will increase fitness in natural environments. Thus, the overall risk for most constructs is expected to be low. If mice were developed to be resistant to pest control measures (pesticides) or to be more disease resistant, then risks would be much higher. However, the use of mice in this way seems quite unlikely.

## The Study of Cell Culture

### The Cell Culture and Growth of Animal Cell

The process of cell culture is technically far more difficult than the culture of bacteria or yeasts; a wide variety of animal and plant cells can be grown and manipulated in culture. Such in vitro cell culture systems have enabled scientists to study cell growth and differentiation, as well as to perform genetic manipulations required to understand gene structure and function.



Under appropriate conditions some specialised cell types can also be grown in culture, allowing their differentiated properties to be studied in a controlled experimental environment. However Animal cell cultures are initiated by the dispersion of a piece of tissue into a suspension of its component cells, which is then added to a culture dish containing nutrient media. Most animal cell types, such as

fibroblasts and epithelial cells, attach and grow on the plastic surface of dishes used for cell culture. Because they contain rapidly growing cells, embryos or tumours are frequently used as starting material. Embryo fibroblasts grow particularly well in culture and consequently are one of the most widely studied types of animal cells. The culture media required for the propagation of animal cells are much more complex than the minimal media sufficient to support the growth of bacteria and yeasts. Early studies of cell culture utilised media consisting of undefined components, such as plasma, serum and embryo extracts. A major advance was thus made in 1955, when Harry Eagle described the first defined media that supported the growth of animal cells. In addition to salts and glucose, the media used for animal cell cultures contain various amino acids and vitamins, which the cells cannot make for them.

The growth media for most animal cells in culture also include serum, which serves as a source of polypeptide growth factors that are required to stimulate cell division. Several such growth factors have been identified. They serve as critical regulators of cell growth and differentiation in multicellular organisms, providing signals by which different cells communicate with each other. For example, an important function of skin fibroblasts in the intact animal is to proliferate when needed to repair damage resulting from a cut or wound. Their division is triggered by a growth factor released from platelets during blood clotting, thereby stimulating proliferation of fibroblasts in the neighbourhood of the damaged tissue. The identification of individual growth factors has made possible the culture of a variety of cells in serum-free media (media in which serum has been replaced by the specific growth factors required for proliferation of the cells in question).

The cells in a primary culture usually grow until they cover the culture dish surface. They can then be removed from the dish and replated at a lower density to form secondary cultures. This process can be repeated many times, but most normal cells cannot be grown in culture indefinitely. For example, normal human fibroblasts can

usually be cultured for 50 to 100 population doublings, after which they stop growing and die. In contrast, cells derived from tumours frequently proliferate indefinitely in culture and are referred to as immortal cell lines. In addition, a number of immortalised rodent cell lines have been isolated from cultures of normal fibroblasts. Instead of dying as most of their counterparts do, a few cells in these cultures continue proliferating indefinitely, forming cell lines like those derived from tumours. Such permanent cell lines have been particularly useful for many types of experiments because they provide a continuous and uniform source of cells that can be manipulated, cloned and indefinitely propagated in the laboratory.

Even under optimal conditions, the division time of most actively growing animal cells is on the order of 20 hours ten times longer than the division time of yeasts. Consequently, experiments with cultured animal cells are more difficult and take much longer than those with bacteria or yeasts. For example, the growth of a visible colony of animal cells from a single cell takes a week or more, whereas colonies of *E. coli* or yeast develop from single cells overnight. Nonetheless, genetic manipulations of animal cells in culture have been indispensable to our understanding of cell structure and function.

## Organ Culture

In this kind of culture the entire embryos organs or tissues are excised from the body either by vivisection or shortly after brain death.

In organ culture:

1. Normal physiological functions are maintained.
2. Cells remain fully differentiated.
3. Scale-up is not recommended.
4. Growth is slow.
5. Fresh explanation is required for every experiment.

## **Tissue Culture**

In this culture, fragments of excised tissue are grown in culture medium.

1. Some normal functions may be maintained.
2. Original organisation of tissue is lost.
3. Better than organ culture for scale-up but not ideal.

## **Cell Culture**

Tissue or outgrowth from an explant is dispersed, mostly enzymatically, into a cell suspension, which may then be cultured as a monolayer or suspension culture.

1. Development of a cell line over several generations
2. Scale-up is possible
3. Cells may lose some differentiated characteristics.

## **Advantages**

- Absolute control of physical environment
- Homogeneity of sample
- Less compound needed than in animal models

## **Disadvantages**

- Hard to maintain
- Only grow small amount of tissue at high cost
- Dedifferentiation
- Instability, aneuploidy

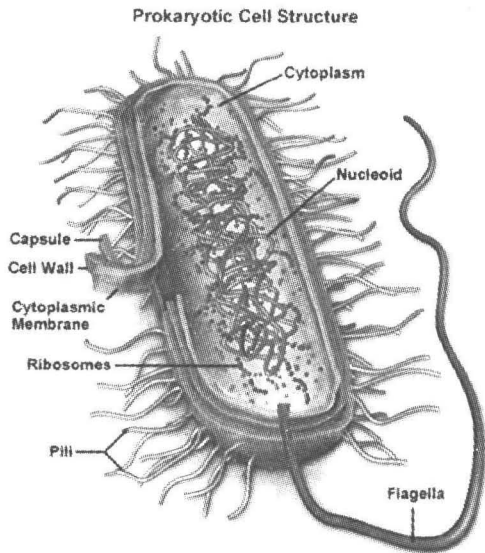
## Procaryotes and Eucaryotes

### Procaryotes

1. Rigid cell wall
2. No nuclear membrane
3. No organelles

### Eucaryotes

1. No cell wall
2. Nuclear membrane
3. Many organelles like;
  - Endoplasmic reticulum
  - Golgi apparatus
  - Mitochondria
  - Lysosome



## Plasma Membrane

### Phospholipids and Proteins

- Phospholipid molecules are arranged in two parallel rows called bilayer
  - Polar heads:
  - Phosphate group+ glycerol
  - Hydrophilic
  - Non polar tails
  - Fatty acid

- Hydrophobic
- Proteins are of two types
- Peripheral :
  - Involved in chemical reactions
  - Can be easily removed
- Integral:
  - Channels for exchange of substances
  - Not easily removed

### Production of Animal Cell Culture

1. Vaccine production
2. Monoclonal antibody production
3. Enzymes and hormones production
4. In vitro skin cell growth: Clinical practice

### **In vitro vs. In vivo**

#### **Environment is different**

1. Cell-cell interaction is reduced in vitro
2. Cell -matrix interactions reduced in vitro
3. Nutritional milieu is changed in vitro
4. Spreading is increased in vitro
5. Migration is increased in vitro
6. Proliferation is increased in vitro

### **Types of Investigations in Cell Culture**

1. Intracellular activity
2. DNA replication and transcription
3. Protein synthesis

4. Energy metabolism
5. Drug metabolism
6. Movement of RNA
7. Translocation of hormone receptor complexes
8. Signal transduction processes
9. Environmental Interactions
10. Nutrition
11. Infection
12. Virally induced transformation
13. Chemically induced transformation
14. Drug action
15. Secretion of specialised products
16. Cell-Cell interaction
17. Embryonic studies
18. Cell population kinetics
19. Cell-cell adhesion
20. Invasion
21. Cell Products
22. Product formation
23. Secretion
24. Genetics
25. Genetic analysis
26. Genetic manipulations
27. Transformation
28. Immortalisation



## Progress in Cell Culture

Two major branches of medical research helped develop the modern cell culture technology

1. Production of viral vaccines
2. Understanding of neoplasia

Other factors

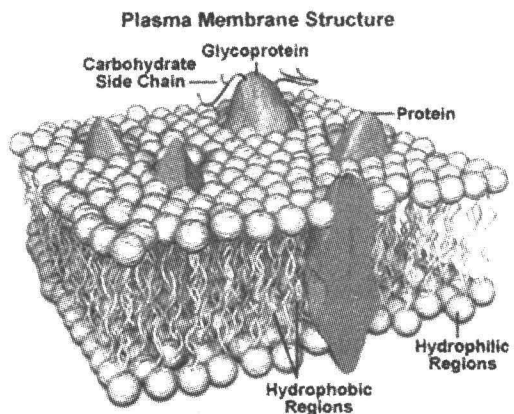
1. Reliable sera and media
2. Use of antibiotics
3. Use of clean air equipment

## Pressure from Animal Right Groups

1. Cell fusion technology
2. Genetic manipulations
3. MAb production and immunological studies
4. Viral infections assayed quantitatively and qualitatively on monolayer of appropriate host cell
5. Genome project

## Properties of Animal cells

1. 10-100 microns
2. Spherical in suspension
3. No cell wall
4. Plasma membrane is thin, fragile and shear sensitive
5. Surface is negatively charged



6. Grown on positively charged surfaces
7. Collagen

## **Downside of Animal Cells**

- Low productivity
- Good producers 100  $\mu\text{g/ml/day}$
- Average producers 20-100  $\mu\text{g/ml/day}$
- Poor producers < 20  $\mu\text{g/ml/day}$
- Slow growth rate
- Low expression rate
- Complexity of growth conditions
- Complexity of media

## **The Method of Gene Cloning**

In cloning technology an exact copy of a living organism is produced. The term may also be used to apply to making a copy of simple cells, a gene or a segment of DNA.. Humans and other mammals use sexual reproduction - the mother's egg is fertilised by the father's sperm - so that the baby has a combination of genes from both parents (one set from each). Cloning is asexual reproduction, because both sets of genes come from one 'parent'. The resulting offspring born from cloning technology has the same genetic makeup as its original parent and is almost like the parent's twins but of a different generation.

Cloning is a controversial technology and the idea of cloning human beings is largely condemned by the worldwide scientific community. However, there are strong advocates for therapeutic cloning. Some commentators believe that human cloning could one day become a reality.

### **Different Cloning Techniques:**

#### **Somatic Cell Nuclear Transfer**

Donor cells or cells from the original organism are grown in the laboratory. The female egg (ovum) is prepared by having its genetic material removed. The selected donor cell is placed next to the empty

ovum and a small electrical current allows the genetic material from the donor egg to fuse into the ovum. The ovum, with its complete set of genes, is 'tricked' into thinking it has been fertilised and so develops into an embryo. The embryo is then implanted into a prepared uterus.

## **Embryo Splitting Techonology**

This is the simplest way to split a fertilised egg in two. Soon after fertilisation, the fertilised egg divides from an embryo. The cells of the embryo are called blastomeres. Using special instruments, the coating of the fertilised egg (the zona pellucida) is taken away. The blastomeres are teased apart and each coated with an artificial zona pellucida, which prompts them to start growing as individual embryos. This technique has been employed for stock breeding. In practice, it is only possible to get a maximum of four embryos using this method.

## **The Technology of Nuclear Transplantation**

This is a process of making multiple copies of the same original cell. The blastomeres from one embryo are fused into prepared egg cells using an electric current. The genetic material can be taken from the body (such as the skin or ear) or from a fertilised ovum.

## **Some of the possible uses of Human Cloning Technology**

Infertility - cloning either the mother or the father may be the only way an infertile couple can have a child under some circumstances.

IVF technology - couples undergoing IVF technology could increase the number of available embryos implanted into the woman's uterus if each fertilised egg was prompted technologically to divide in two and become twins.

Single women and lesbians - women who desire children but don't have or don't want, male partners could clone themselves.

Organ transplants - the major obstacles facing organ transplant are the lack of available donor organs and the problem of rejection. Theoretically, cloning techniques could produce made-to-order organs that the recipient's immune system wouldn't reject. For example, a person with organ failure may be cloned so that their 'twin' can provide the replacement tissue. This has been termed therapeutic cloning.

## **A Specific Gene Cloning**

We have seen the generic approaches to create recombinant DNA. However, a geneticist is interested in isolating and characterising some particular gene of interest, so the procedures must be tailored to isolate a specific recombinant DNA clone that will contain that particular gene. The details of the process differ from organism to organism and from gene to gene. An important initial factor is the choice of an appropriate vector for the job at hand.

## **The Cloning Vector**

The small molecule, the ideal vector should facilitate manipulation.. It must be capable of prolific replication in a living cell, thereby enabling the amplification of the inserted donor fragment. Another important requirement is to have convenient restriction sites that can be used for insertion of the DNA to be cloned. Unique sites are most useful because then the insert can be targeted to one site in the vector. It is also important to have a method for easily identifying and recovering the recombinant molecule. Numerous cloning vectors are in current use and the choice between them often depends on the size of the DNA segment that needs to be cloned and on the intended application for the cloned gene. We shall consider several commonly used types.

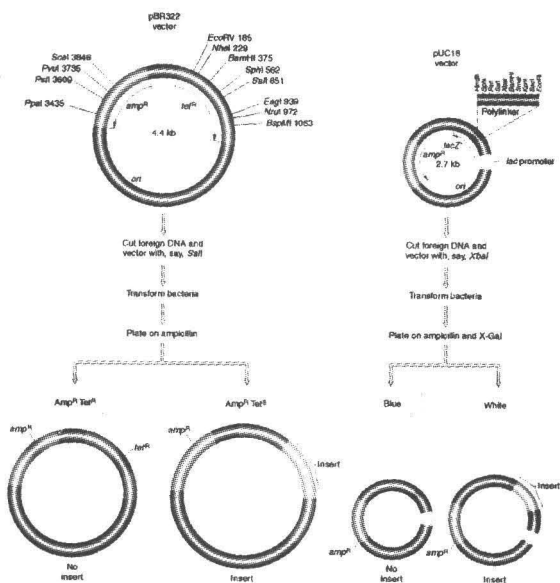
## **The Bacteria Plasmids**

Many different types of plasmids have been found in bacteria. They are small circular DNA molecules that are not only distinct from the

main bacterial chromosome, but also additional to it. They replicate their DNA independently of the bacterial chromosome. The distribution of any one plasmid within a species is generally sporadic; some cells have the plasmid, whereas others do not. The F plasmid confers certain types of conjugative behaviour to cells of *E. coli*. The F plasmid can be used as a vector for carrying large donor DNA inserts. However, the plasmids that are routinely used as vectors are those that carry genes for drug resistance. The drug-resistance genes are useful because the drug-resistant phenotype can be used to select not only for cells transformed by plasmids, but also for vectors containing recombinant DNA.

Plasmids are also an efficient means of amplifying cloned DNA because there are many copies per cell, as many as several hundred for some plasmids.

Observe the picture see the two plasmid vectors. These vectors are derived from natural plasmids, but both have been genetically modified



for convenient use as recombinant DNA vectors. Plasmid pBR322 is simpler in structure; it has two drug resistance genes, *tet<sup>r</sup>* and *amp<sup>r</sup>*. Both genes contain unique restriction target sites that are useful in cloning. For example, donor DNA could be inserted into the *tet<sup>r</sup>* gene. A successful insertion will split and inactivate the *tet<sup>r</sup>* gene, which will then no longer confer tetracycline resistance and the cell will be sensitive to that drug. Therefore, the cloning procedure is to mix the samples of cut plasmid and donor DNA, transform bacteria and select first for ampicillin-resistant colonies, which must have been successfully transformed by a plasmid molecule. Of the *Amp<sup>r</sup>*

colonies, only those that prove to be tetracycline sensitive have inserts, in other words, the Amp<sup>R</sup> Tet<sup>S</sup> colonies are the ones that contain recombinant DNA. Further experiments are needed to find the clones with the specific insert required.

The pUC plasmid is a more advanced vector, whose structure allows direct visual selection of colonies containing vectors with donor DNA inserts. The key element is a small part of the *E. coli* galactosidase gene. Into this region has been inserted a piece of DNA called a polylinker or multiple cloning site, which contains many unique restriction target sites useful for inserting donor fragments. The polylinker is in frame translationally with the galactosidase fragment and does not interfere with its translation. The transformation protocol uses recipient cells that contain a galactosidase gene lacking the fragment present on the plasmid. An unusual type of complementation takes place in which the partial proteins encoded by the two fragments unite to form a functional galactosidase. A colourless substrate for galactosidase called X-Gal is added to the medium and the functional enzyme converts this substrate into a blue dye, which colours the colony blue. If donor DNA is inserted into the polylinker, the enzyme fragment borne on the vector is disrupted, no complete galactosidase protein is formed and the colony is white. Hence, selection for white Amp<sup>R</sup> colonies selects directly for vectors bearing inserts and such colonies are isolated for further study.

Small plasmids that contain large inserts of foreign DNA tend to spontaneously lose the insert; therefore, these plasmids are not useful for cloning DNA fragments larger than 20 kb.

## **Viral Vectors**

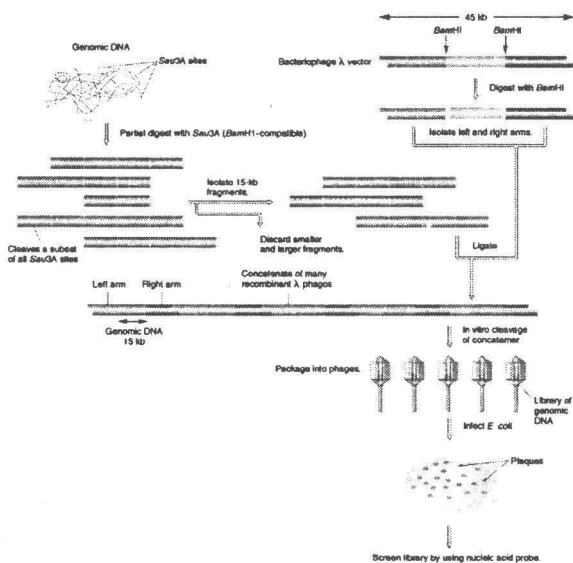
The gene or genes of interest are incorporated into the genome of a virus in the process of Viral Vectors. This offers many advantages for cloning and the subsequent applications of cloned genes. Because viruses infect cells with high efficiency, the cloned gene can be introduced into cells at a significantly higher frequency than by simple transformation. Some viral vectors are specialised for producing high

levels of proteins encoded by the cloned genes, as exemplified by the use of insect baculovirus to express foreign proteins in a eukaryotic cell system. Other viral vectors, such as the bacterial M13-based vectors, are designed to facilitate sequencing and the generation of mutations in cloned genes. Vectors derived from retroviruses can effect the stable integration into mammalian chromosomes of cloned DNA, allowing continued expression of the gene. Viral vectors are also the vehicles of choice for gene-therapy strategies.

Some examples of viral vectors used in bacteria:

## Phage Lambda

Phage is a convenient cloning vector for several reasons. First, phage heads will selectively package a chromosome about 50 kb in length and, as will be seen, this property can be used to select for molecules with inserts of donor DNA. The central part of the phage genome is not



required for replication or packaging of DNA molecules in *E. coli*, so the central part can be cut out by using restriction enzymes and discarded. The two 'arms' are ligated to restriction-enzyme-cut donor DNA. The chimeric molecules can be either introduced into *E. coli* directly by transformation or packaged into phage heads in vitro. In the in vitro system, DNA and phage-head components are mixed together and infective phases form spontaneously. In either method, recombinant molecules with 10- to 15-kb inserts are the ones that



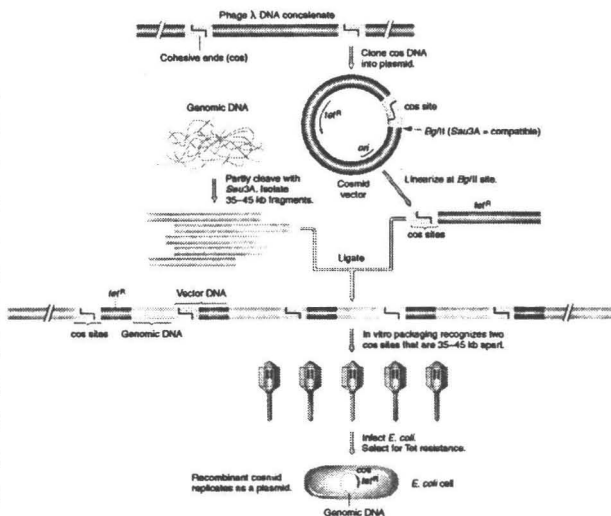
will be most effectively packaged into phage heads, because this size of insert substitutes for the deleted central part of the phage genome and brings the total molecule size to 50 kb. Therefore the presence of a phase plaque on the bacterial lawn automatically signals the presence of recombinant phage bearing an insert. A second useful property of a phage vector is that recombinant molecules are automatically packaged into infective phase particles, which can be conveniently stored and handled experimentally.

## Single-Stranded Phases

Some phases contain only single-stranded DNA molecules. On infection of bacteria, the single infecting strand is converted into a double-stranded replicative form, which can be isolated and used for cloning. The advantage of using these phases as cloning vectors is that single-stranded DNA is the very substrate required for the Sanger DNA-sequencing technique currently in widespread use. Phage M13 is the one most widely used for this purpose.

## Cosmids

Cosmid DNA can be packaged into phage particles by using the *in vitro* system. These vectors are hybrids of phases and plasmids and their DNA can replicate in the cell like that of a plasmid or be packaged like that



of a phage. However, cos-mids can carry DNA inserts about three times as large as those carried by itself (as large as about 45 kb). The

key is that most of the phage structure has been deleted, but the signal sequences that promote phage-head stuffing (cos sites) remain. This modified structure enables phage heads to be stuffed with almost all donors DNA.

## Vector Expressions

By detecting its protein product expressed in the bacterial cell one can detect a specific cloned gene. Therefore, in these cases, it is necessary to be able to express the gene in bacteria, that is, to transcribe it and translate the mRNA into protein. Most cloning vectors do not permit expression of cloned genes, but such expression is possible if special vectors are used. However, because bacteria cannot process introns, the cloned sequences must be stripped of introns. The cloned gene is inserted next to appropriate bacterial transcription and translation start signals. Some expression vectors have been designed with restriction sites located just next to a lac regulatory region. These restriction sites permit foreign DNA to be spliced into the vector for expression under the control of the lac regulatory system.

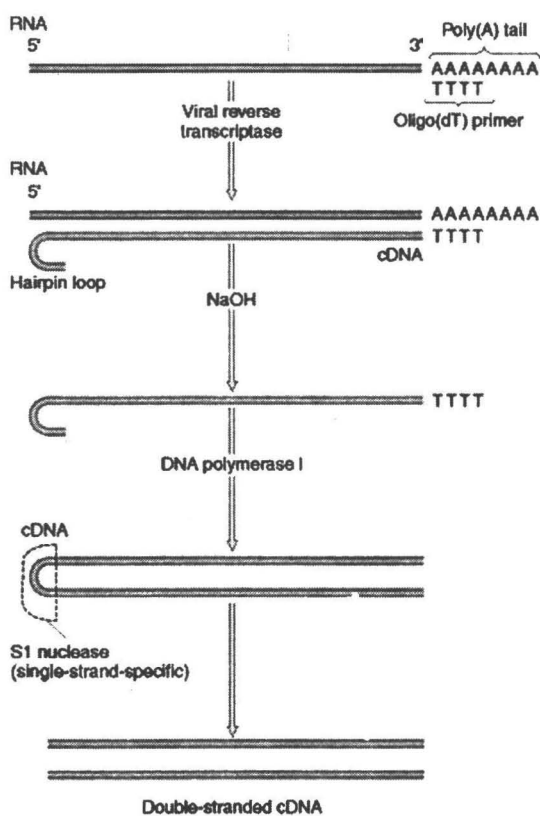
## The DNA Library

The most important goal of recombinant DNA technology is to clone a particular gene or other genomic fragment of interest. The approach used to clone a specific gene depends to a large degree on the gene in question and on what is known about it. Generally, the procedures start with a sample of DNA such as eukaryotic genomic DNA. The next step is to obtain a large collection of clones made from this original DNA sample. The collection of clones is called a DNA library. This step is sometimes referred to as 'shotgun' cloning because the experimenter clones a large sample of fragments and hopes that one of the clones will contain a 'hit' the desired gene. The task then is to find that particular clone.

According to the use of vectors and according to the source of DNA we find different types of libraries. Different cloning vectors carry different amounts of DNA, so the choice of vector for library

construction depends on the size of the genome (or other DNA sample) being made into the library. Plasmid and phage vectors carry small amounts of DNA, so these vectors are suitable for cloning genes from organisms with small genomes. Cosmids carry larger amounts of DNA and other vectors such as YACs and BACs carry the largest amounts of all. Ease of manipulation is another important factor in choosing a vector. A phage library is a suspension of phages. A plasmid or a cosmid library is a suspension of bacteria or a set of defined bacterial cultures stored in culture tubes or microtiter dishes.

Another important decision is whether to make a genomic library or a cDNA library. The cDNA or complementary DNA is synthetic DNA made from mRNA with the use of a special enzyme called reverse transcriptase originally isolated from retroviruses. With the use of an mRNA as a template, reverse transcriptase synthesises a single-stranded DNA molecule that can then be used as a template for double-stranded



DNA synthesis. Because it is made from mRNA, cDNA is devoid of both upstream and downstream regulatory sequences and of introns. Therefore cDNA from eukaryotes can be translated into functional protein in bacteria an important feature when expressing eukaryotic genes in bacterial hosts.

Choosing between cDNA and genomic DNA depends on the situation. If a specific gene that is active in a specific type of tissue in a plant or animal is being sought, then it makes sense to use that tissue to prepare mRNA to be converted into cDNA and then make a cDNA library from that sample. This library should be enriched for the gene in question. A cDNA library is based on the regions of the genome transcribed, so it will inevitably be smaller than a complete genomic library, which should contain all of the genome. Although genomic libraries are bigger, they do have the benefit of containing genes in their native form, including introns and regulatory sequences. If the purpose of constructing the library is a prelude to cloning an entire genome, then a genomic library is necessary. In some cases, it is possible to narrow the genomic fraction used in library construction to more easily detect the desired gene. This approach is possible if the experimenter already knows which chromosome contains the gene. One technique used in mammalian molecular genetics is to sort the chromosomes with an instrument called a flow cytometer. A suspension of chromosomes is passed through the apparatus, which sorts the chromosomes according to size. The appropriate chromosomal fraction is then used to make the library.

Electrophoresis is a general technique that fractionates nucleic acids or proteins according to size on gels under the influence of a strong electric field. This type of procedure separates shorter DNA fragments. PFGE is a specialised type of electrophoresis useful for very long DNA molecules. It uses several oscillating electric fields oriented in several different directions. These electric fields enable large DNA molecules such as whole chromosomes to snake through the gel to different positions according to their size. The appropriate chromosome can be identified on the gel by probing with a chromosome-specific probe. Then the desired chromosome can be cut out, eluted from the gel and used to make a chromosome-specific library.

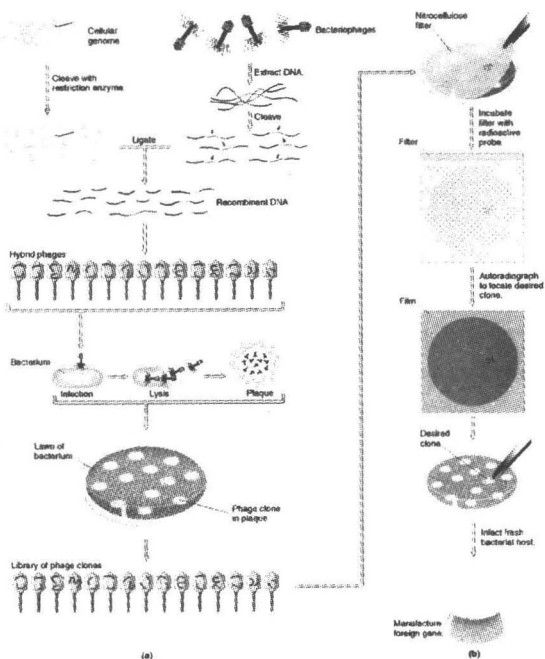
How can an experimenter determine whether a library is large enough to contain any one unique sequence of interest with a reasonable degree of certainty? There are formulas for calculating the minimum number of clones needed, but a rough idea of the general order of

magnitude of the library can be obtained simply by taking the total genome size and dividing by the average size of the inserts carried by the vector being used. Generally, this number will be at least doubled, but it does provide a rough estimate of the magnitude of the job of library construction.

The library, which might contain as many as hundreds of thousands of cloned fragments, must be screened to find the recombinant DNA molecule containing the gene of interest. Such screening is accomplished by using a specific probe that will find and mark the clone for the researcher to identify.

Broadly speaking, there are two types of probes: those that recognise DNA and those that recognise protein.

DNA, when denatured (made single stranded by unwinding the two halves of the double helix), will find and bind to other similar denatured DNAs in the library. Identification of a specific clone in a library is a two-step procedure. First, colonies or plaques of the library on a petri plate are transferred to an absorbent membrane (often nitrocellulose) by simply laying the membrane



on the surface of the medium. The membrane is peeled off and colonies or plaques clinging to the surface are lysed in situ and the DNA denatured. The next step is to bathe the membrane with a solution of a probe that is specific for the DNA being sought. The probe must be

labelled either with radioactivity or with a fluorescent dye. Generally, the probe is itself a cloned piece of DNA that has a sequence homologous to the desired gene. The probe DNA must be denatured, it will then bind only to the DNA of the clone being sought. The position of a positive clone will become clear from the position of the concentrated label, often as a spot on an autoradiogram.

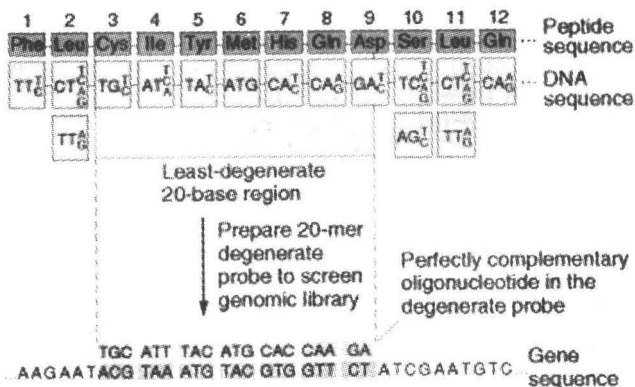
In mammalian reticulocytes, 90 percent of the mRNA is known to be transcribed from the globin gene. So reticulocytes would be a good source of mRNA for making a cDNA probe to find a genomic globin gene. In this case, a genomic library would be probed. The need for this kind of analysis depends on which questions are to be asked about the gene. If only the transcribed sequence is of interest, then the cDNA clone itself could provide that information just as well. However, if introns and control regions are needed, the genomic clone must be obtained.

A homologous gene from a related organism is another source of DNA a probe. For example, if a certain gene has been cloned in the ascomycete fungus *Neurospora*, then it is very likely that this gene can be used as a probe to find the homologous gene in the related fungus *Podospora*. This method depends on the evolutionary conservation of DNA sequences through time. Even though the probe DNA and the DNA of the desired clone might not be identical, they are often similar enough to promote hybridisation. The method is jokingly called 'clone by phone' because, if you can telephone a colleague who has a clone of your gene of interest but from a related organism, then your job of cloning is made relatively easy.

If the protein product of the gene of interest is known and an amino acid sequence has been obtained probe DNA can be synthesised. Synthetic DNA probes are designed on the basis of knowledge of the genetic code, so an amino acid sequence merely has to be translated backward to obtain the DNA sequence that encoded it. However, because of the redundancy of the code in other words, the fact that most amino acids are coded by more than one codon several possible DNA sequences could have encoded the protein in question. To get around this problem, a short stretch of amino acids with minimal

redundancy is selected. The nucleotide sequence is calculated by using the codon dictionary.

The chemical DNA synthesising reaction is a step-by-step process, so, wherever in the sequence there are alternative nucleotides, a



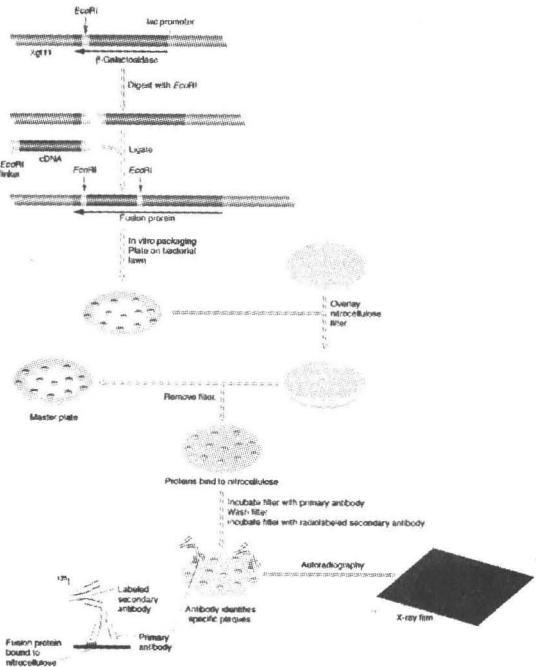
mixture of those alternative nucleotides is fed into the reaction and all possible DNA strands are synthesised. An example shows there are five positions of redundancy, showing 2, 3, 2, 2 and 2 alternatives, respectively. The reaction would make  $2 \times 3 \times 2 \times 2 \times 2 = 48$  oligonucleotide strands at the same time. This 'cocktail' of oligonucleotides would be used as a probe. The correct strand within this cocktail would find the gene of interest. Twenty nucleotides embody enough specificity to find one unique DNA sequence in the library.

Additionally, free RNA can be radioactively labelled and used as a probe. This use of labelled free RNA as a probe is possible only when a relatively pure population of identical molecules of RNA, such as rRNA or fractionated tRNAs, can be isolated.

## Proteins Probe

When the protein product of a gene is known and isolated in pure form, then this protein can be used to detect the clone of the corresponding gene in a library. An antibody to the protein is prepared and this antibody is used to screen an expression library. These libraries are made by using expression vectors designed to express high levels of a specific bacterial protein. To make the library, cDNA is inserted into the vector in frame with the bacterial protein and the cells will

make a fusion protein. A membrane is laid over the surface of the medium and removed with an imprint of colonies. It is dried and bathed in a solution of the antibody. Positive clones are revealed by making an antibody to the first antibody, the second antibody is labeled by a radioactive isotope or a chemical that will fluoresce or become a coloured dye. By detecting the correct protein, the antibody effectively identifies the clone containing the gene that must have synthesised that protein.



From tyrosinase producing cells, mRNA was isolated and used to make cDNA. This cDNA was used to make an expression-vector library. The library was probed with the antibody to tyrosinase and several positive clones were detected. The cDNA in the positive clone was sequenced and found to contain a gene whose exons total 1590 nucleotide pairs. The cDNA was used to probe a library of human genomic DNA and, in this process, the intact tyrosinase gene was found. It proved to have five exons and four introns.

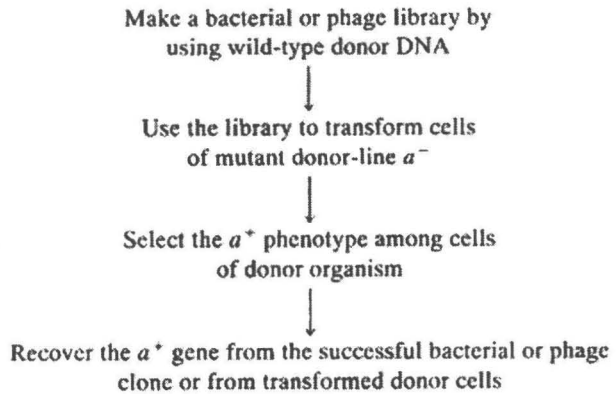
## Finding specific clones by functional complementation

Through their ability to confer a missing function on a mutant line of the donor organism, specific clones in a bacterial or phage library can be detected. This acts as the transformation recipient. This procedure



is called functional complementation.

This method depends on the ability to transform the donor organism, often a eukaryote. We have already considered tr-



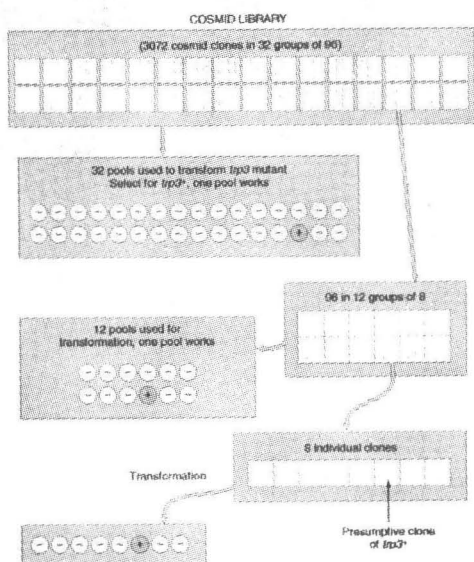
ansformation in prokaryotes, but eukaryotes can be transformed, too. The procedure differs among eukaryotes, but generally some special treatment of recipient cells is required. For example, to transform fungi, generally the cell walls must be removed enzymatically. Let's assume that we have isolated a mutant that is relevant to some biological process that interests us. For the present purpose, we will assume that it is an auxotrophic mutation in a fungus. We shall use DNA from the library to transform the auxotrophic mutant strain and then plate these recipient fungal cells on minimal medium. Fungal cells that contain the wild-type allele (from the wild-type culture used to make the library) will transform the auxotroph to prototrophy and allow growth on minimal medium. Because this transformation method works transforming fragment functionally complements and the deficiency is caused by the mutant allele in the recipient.

It might seem at first that this view of complementation is not the same as the one developed in that is, the production of a wild-type phenotype from the union of two mutant genomes. However, the transforming vector contributes something that the recipient genome lacks (the wild-type allele being sought) and the recipient genome contributes something that the vector lacks (the entire remainder of the genome), so a type of complementation is accomplished.

If the transformation recipient is an organism in which plasmid vectors replicate autonomously (mainly bacteria and yeasts), then the transforming insert can be recovered simply by isolating the plasmid.

However, as we shall see, in most eukaryotic organisms, the bacterial or phage vector cannot replicate and must insert into the genome to achieve stable transformation. In these cases, the transforming fragment is relatively inaccessible and must be retrieved from the successful clone in the library. This method uses a library in which the clones are laid out as a collection of numbered bacterial cultures in tubes or microtiter dishes. DNA is isolated in bulk from all the strains in specific subsets of the library and transformation is attempted. By a process of narrowing down the library subsets that successfully transform, the clone with the wild-type allele can be identified.

Observe the picture this process is illustrated. This process is an example of *Neurospora trp3* gene. In this case, a cosmid library was used. The cosmid must also carry a marker gene that can be used to select for successful transformants of the fungus. A gene for hygromycin resistance is commonly used in fungi, which are normally sensitive to this drug. Subsets of the cosmid library made from wild-



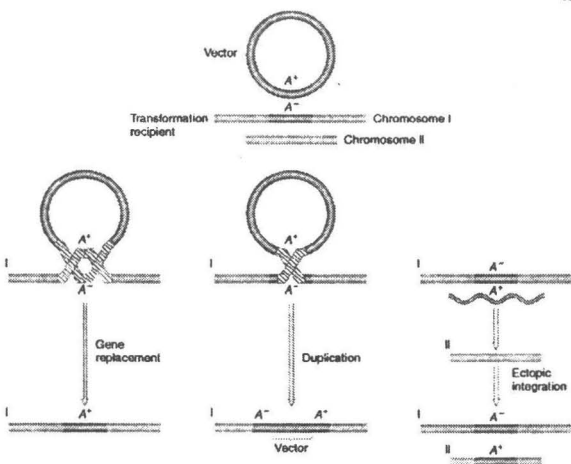
type *Neurospora* DNA were used to transform *trp3* mutant cells and *trp3*<sup>+</sup> clones were selected by plating transformed cells on medium containing hygromycin but lacking tryptophan. Colonies that grow are likely to contain the *trp3*<sup>+</sup> allele and are isolated from the plate.

In most cases, transformants are found to contain the vector carrying the wild-type allele inserted into one of the recipient's chromosomes at a location that is different from the mutant locus in the recipient. This is called ectopic insertion. Less commonly, the transforming wild-

type allele replaces the resident auxotrophic mutation by a double-crossover-like process.

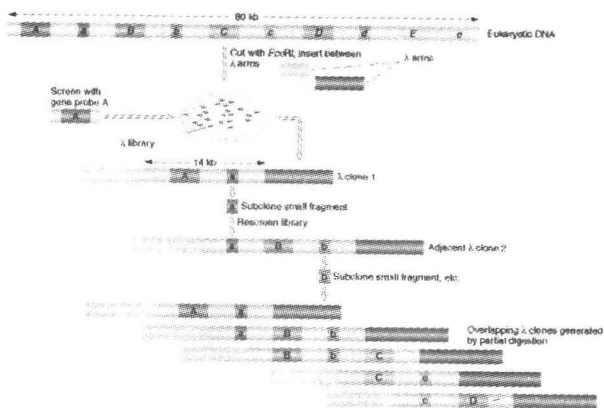
If a eukaryotic gene is cloned on a prokaryotic vector but a specific eukaryotic sequence is known that can act as an origin of replication, this se-

quence can be added to the vector. Then the vector will be able to replicate in both bacterial and eukaryotic cells and insertion into the chromosome is not essential. These types of vectors are called shuttle vectors because they can be moved back and forth between different hosts. Without an origin of replication, the donor DNA must integrate into the eukaryotic chromosome to effect stable transformation.



## Positional Cloning

Information about a gene's position in the genome can be used to circumvent the hard work of assaying an entire library to find the clone of interest. Positional cloning is a term that can be



applied to any method that makes use of such information. Often both probing and complementation are part of positional cloning. A

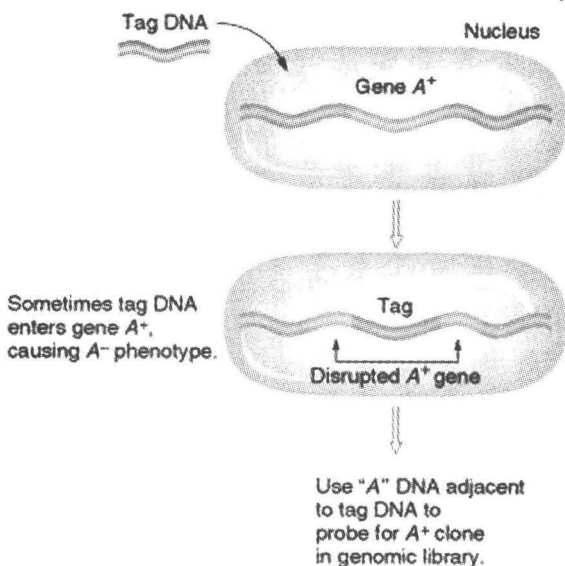
common starting point is the availability of another cloned gene or other marker known to be closely linked to the gene being sought. The linked marker acts as the departure point in a process, called chromosome walking, that will terminate at the target gene. Summarises the procedure of chromosome walking. End fragments of a clone of the linked marker are used as probes to select other clones from the library. These probes will detect clones of DNA regions that overlap with the initial clone. Restriction maps are made of the DNA of this second set of clones and, again, outward fragments are used for a new round of selection of overlapping clones from the library. Hence the walking process moves outward in two directions from the start site. Each clone can be sequenced or otherwise tested, depending on the intent of the exploration.

The availability of a large number of neutral DNA markers (restriction fragment length polymorphisms) dispersed throughout most genomes has provided many useful start points. Positional cloning has been particularly useful for cloning human genes, many of which have no known biochemical function and cannot be easily selected by functional complementation. The human gene for cystic fibrosis, mentioned at the beginning of the chapter, was cloned by chromosome walking and we shall examine its cloning. For any case of chromosome walking, there must be some type of criterion to assess each step of the walk for the gene of interest and these criteria depend on the individual gene concerned.

## Gene Cloning by Tagging

This method of tagging induces a mutation with the use of a specific piece of DNA as an inspectional mutagen. The specific sequence is then used as a tag to recover the gene. The approach is summarised. One type of tag is transforming DNA. When exogenous DNA is added by transformation or by other methods such as injection, it can integrate into the genome and become part of the chromosome. Ectopic integration is random throughout the genome and apparently no segment of chromosomal DNA is immune to integration. When integration takes place within or near a gene, the integrating fragment

acts as a mutagen, disrupting the function of the gene. This property can be used to good advantage. Suppose that we use a specific cloned gene  $x^+$  and transform  $x$  cells of the donor organism into  $x^+$ . Many of the  $x^+$  transformants will be mutant for the genes into which the transforming DNA has inserted



ectopically. A subset of such  $x^+$  cells will be mutant for the target gene  $a^+$ , the gene of interest and will be of phenotype  $a$ . Hence among the  $x^+$  transformants, a phenotypes are identified. The next step is to cross the transformants to determine if a phenotype segregates with  $x^+$ . If it does, the mutation is likely to have been caused by the integration of the fragment containing  $x^+$ . The DNA of this mutant line is used to construct a library and gene  $x^+$  can be used as a probe to recover the clone of the disrupted  $a$  gene. To recover the intact wild-type  $a$  gene, a fragment of the disrupted  $a$  gene sequence is used in another round of probing, this time with a wild-type library.

A similar approach uses transposons as tags. Transposons are naturally mobile DNA fragments found in many organisms. When they move, they can insert anywhere in the genome. If they insert into or near a gene, they can create a null mutation. (In a line containing an active transposon, mutants for the desired gene are selected. Many of these mutants will have been caused by the insertion of the transposon. This mutant line is used to make a library. A cloned part of the transposon DNA can then be used as a tag to recover the gene, in a manner similar to that as shown in the above figure.

## The Features of a Cloning Vector

A DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of itself and the foreign DNA. The molecular analysis of DNA has been made possible by the cloning of DNA. The two molecules that are required for cloning are the DNA to be cloned and a cloning vector.

## The features of all Cloning Vectors

- Sequences that permit the propagation of itself in bacteria (or in yeast for YACs)
- A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes
- A method of selecting for bacteria (or yeast for YACs) containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance

## Types of Cloning Vectors

- **Plasmid** - an extrachromosomal circular DNA molecule that autonomously replicates inside the bacterial cell; cloning limit: 100 to 10,000 base pairs or 0.1-10 kilobases (kb)
- **Phage** - derivatives of; linear DNA molecules, whose region can be replaced with foreign DNA without disrupting its life cycle; cloning limit: 8-20 kb
- **Cosmids** - an extrachromosomal circular DNA molecule that combines features of plasmids and phage; cloning limit - 35-50 kb
- **Bacterial Artificial Chromosomes (BAC)** - based on bacterial mini-F plasmids. Cloning limit: 75-300 kb
- **Yeast Artificial Chromosomes (YAC)** - an artificial chromosome that contains telomeres origin of replication, a yeast centromere and a selectable marker for identification in yeast cells; cloning limit: 100-1000 kb

## How to Clone with any Vector

- Prepare the vector and DNA to be cloned by digestion with restriction enzymes to generate complementary ends
- Legate the foreign DNA into the vector with the enzyme DNA ligase
- Introduce the DNA into bacterial cells (or yeast cells for YACs) by transformation
- Select cells containing foreign DNA by screening for selectable markers (usually drug resistance)

## Cloning cDNA

The cloning that has been described here will work for any random piece of DNA. But since the goal of many cloning experiments is to obtain a sequence of DNA that directs the production of a specific protein, any procedure that optimises cloning will be beneficial. One such technique is cDNA cloning. The principle behind this technique is that an mRNA population isolated from a specific developmental stage should contain mRNAs specific for any protein expressed during that stage. Thus, if the mRNA can be isolated, the gene can be studied. mRNA cannot be cloned directly, but a DNA copy of the mRNA can be cloned. (In this regard, the term cDNA is short for “copy DNA”.) This conversion is accomplished by the action of reverse transcriptase and DNA polymerase. The reverse transcriptase makes a single-stranded DNA copy of the mRNA. The second DNA strand is generated by DNA polymerase and the double-stranded product is introduced into an appropriate plasmid or lambda vector.

## Clone Library Screening

Cloning mRNA through a cDNA intermediate generates a cDNA library that represents the mRNA from that specific developmental stage. In contrast, if you clone total human DNA for example into a lambda or cosmid vector, you obtain a genomic library that contains (with a probability of 95-99%) all the sequences from the human

genome or DNA complement. Lambda or cosmid libraries are typically used for genomic libraries because you generally can clone an entire gene containing both the coding sequence and regulatory elements on a single clone.

One of the key elements required to identify a gene during cloning is a probe. A probe is normally a cloned piece of DNA that contains a portion of the sequence for which you are searching. You typically will make the probe radioactive and add it to a solution. Filters containing immobilised clones are then bathed in the solution. The principal behind this step is that the probe will bind to any clone containing sequences similar to those found on the probe. This binding step is called hybridisation.

- **Probe** - a nucleic acid (usually DNA) that is complementary to a specific gene or nucleic acid sequence of interest; when a cDNA library is being screened an antibody can be used to identify the protein that is being expressed by the insert of the clone
- **Homologous Probe** - a probe that is exactly complementary to the nucleic acid sequence for which you are searching; ex., a human cDNA used to search a human genomic library
- **Heterologous Probe** - a probe that is similar to, but not exactly complementary to the nucleic acid sequence for which you are searching; ex., a mouse probe used to search a human genomic library

## Screening a cDNA or Genomic Library

- Immobilise members of the library onto a nylon membrane and denature them so that they are single-stranded
- Prepare a radiolabelled probe and denature it to make it single-stranded
- Hybridise the probe to the library of clones
- Wash the excess probe and expose an X-ray film
- Isolate the positive clone and analyse



The hybridisation step is performed at a non-stringent temperature that ensures the probe will bind to any clone containing a similar sequence. At the same time some non-specific hybridisation will occur because some of the clones will contain limited, but not significant similarity to the probe. The washing step is performed at a stringent temperature that is high enough to wash the probe off all clones to which it has bound in a non-specific manner. But it is important that the temperature is not so high that it washes the probe off of clones that contain sequences that are similar or identical to the probe itself. Therefore, consideration about the source of the probe (homologous or heterologous) determine the temperature at which the washing step is performed.

## Some Myths and Facts about Animal Biotechnology

**Myth:** Only humans can benefit from medical biotechnology.

**Fact:** Biotechnology-based animal health products and services are estimated to grow to \$5.1 billion by 2005. According to USDA, there are 111 licensed biotech products for animals. These products include veterinary vaccines, biologics and diagnostic kits. The animal health industry invests more than \$400 million a year in research and development. Current sales of biotech-based products for use in animal health generate \$2.8 billion (out of a total market for animal health products of \$18 billion).

**Myth:** Pets do not benefit from biotechnology at all.

**Fact:** Companion animals, better known as pets, benefit greatly from vaccines and diagnostic tests based on biotechnology. Biotech-based products to treat heartworm, arthritis, parasites, allergies and heart disease, as well as vaccines for rabies and feline HIV are used daily by veterinarians. Gene therapy has been used to help restore sight to blind dogs, as well as for melanoma, canine lymphoma and bone cancer. Also, some biotechnology companies are able to sequence the DNA of purebred animals, such as dogs, for identification purposes.

**Myth:** Transgenic and cloned animals are different from normal animals.

**Fact:** Studies and experience have shown that biotech animals are animals that eat, drink and behave similarly to their conventional counterparts.

**Myth:** Animals cannot benefit from biotechnology.

**Fact:** Biotechnology provides new tools for improving animal health and increasing livestock and poultry productivity. These improvements come from the enhanced ability to detect, treat and prevent diseases and other problems. Additionally, feed from biotech crops are better designed to meet the dietary needs of different farm animals. Animal breeding techniques such as in vitro fertilisation, artificial insemination, genetic counselling and cloning can also significantly improve animal breeding programs and decrease the occurrence of hereditary diseases.

**Myth:** Biotech will cause disease outbreaks such as avian flu, mad cow disease and West Nile virus in animals an insect, which will be, transferred to humans.

**Fact:** Diseases such as avian flu, mad cow disease and West Nile virus are not related to or caused by biotechnology. In fact, researchers are working to find ways to apply biotechnology to eliminate some of these diseases. Scientists today have created biotech cows that may be resistant to mad cow disease and some research has been done to develop biotech mosquitoes that will no longer infect humans with malaria and other blood-borne diseases.

**Myth:** Organ transplants from animals are an unreal fantasy.

**Fact:** Xenotransplantation or organ transplants from one species to another, could happen in our lifetime. We know this because doctors already use valves from pigs' hearts for human heart valve transplants and pigs' skins for skin grafts for human burn victims. Although organ transplants are much more complicated, much work is being done to decrease the human rejection of pig organs and make them a viable source for organ transplants.

**Myth:** We are just exploiting animals by applying biotechnology to them.

**Fact:** The health and well being of all animals can benefit from biotechnology. The health of companion animals can be significantly improved through the use of biotech vaccines, such as the rabies vaccine and new diagnostic tests that can identify things such as feline HIV. Domesticated farm animals can greatly benefit from biotechnology through vaccines and diagnostic tests. Improved breeding programs enhanced by biotechnology can drastically improve herd health by eliminating hereditary diseases. Reproduction and breeding techniques influenced by biotechnology, such as in vitro fertilisation, artificial insemination, genetic counselling and cloning animals. While shortened telomere lengths were seen in one sheep (“Dolly”) cloned from adult somatic cells. Telomere lengths apparently are normal in cattle cloned from adult cells.

## Transgenic Animals

The term transgenic animal refers to an animal in which there has been a deliberate modification of the genome - the material responsible for inherited characteristics - in contrast to spontaneous mutation (FELASA September 1992, revised February 1995). Foreign DNA is introduced into the animal, using recombinant DNA technology and then must be transmitted through the germ line so that every cell, including germ cells, of the animal contain the same modified genetic material.

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to a structural gene, the DNA usually includes other sequences to enable it

- To be incorporated into the DNA of the host and
- To be expressed correctly by the cells of the host.
- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesise human proteins in the 'white' of the eggs.

These animals should eventually prove to be valuable sources of proteins for human therapy.

In July 2000, researchers from the team that produced Dolly reported

success in producing transgenic lambs in which the transgene had been inserted at a specific site in the genome and functioned well.

Transgenic mice have provided the tools for exploring many biological questions.

An example:

Normal mice cannot be infected with poliovirus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

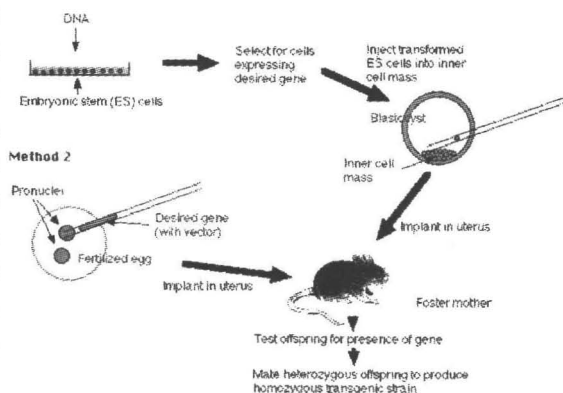
- Can be infected by polio virus and even
- Develop paralysis and other pathological changes characteristic of the disease in humans.

Two methods of producing transgenic mice are widely used:

- Transforming embryonic stem (ES) cells (ES cells) growing in tissue culture with the desired DNA,
- Injecting the desired gene into the pronucleus of a fertilised mouse egg.

## The Embryonic Stem Cell Method (Method '1')

Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, including its gametes.



1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

- The structural gene you desire (e.g., the insulin gene)
- Vector DNA to enable the molecules to be inserted into host DNA molecules
- Promoter and enhancer sequences to enable the gene to be expressed by host cells

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells. [Method]

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

- Prepare a pseudopregnant mouse (by mating a female mouse with a vasectomised male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they implant successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10-20% will have it and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1:4 that will be homozygous for the transgene.
- Mating these will find the transgenic strain.

## **The Pronucleus Method (Method ‘2’)**

1. Prepare your DNA as in Method 1
2. Transform fertilised eggs
  - Harvest freshly fertilised eggs before the sperm head has become a pronucleus.
  - Inject the male pronucleus with your DNA.
  - When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.
3. Implant the embryos in a pseudopregnant foster mother and proceed as in Method 1.

### **An Example**

This image shows a transgenic mouse (right) with a normal littermate (left). The giant mouse developed from a fertilised egg transformed with a recombinant DNA molecule containing:

- The structural gene for human growth hormone
- A strong mouse gene promoter

The levels of growth hormone in the serum of some of the transgenic mice were several hundred times higher than in control mice.

### **Random vs. Targeted Gene Insertion**

The early vectors used for gene insertion could and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that

- restores function in a mutant animal or
- knocks out the function of a particular locus.

In either case, targeted gene insertion requires

- The desired gene

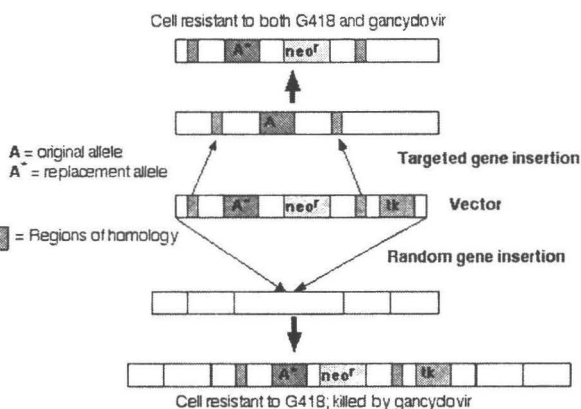
- neo, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells;
- tk, a gene that encodes thymidine kinase, an enzyme that phosphorylates the nucleoside analog gancyclovir. DNA polymerase fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So gancyclovir kills cells that contain the tk gene.

## Step 1

Treat culture of ES cells with preparation of vector DNA.

### Results:

- Most cells fail to take up the vector, these cells will be killed if exposed to G 418.
- In a few cells: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the tk gene, is inserted into host DNA. These cells are resistant to G 418 but killed by gancyclovir.
- In still fewer cells: homologous recombination occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome and the region between these homologous sequences replaces the equivalent region in the host DNA.



## Step 2

Culture the mixture of cells in medium containing both G 418 and gancyclovir.



- The cells (the majority) that failed to take up the vector are killed by G 418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the tk gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).

### Step 3

Inject these into the inner cell mass of mouse blastocysts.

## Knockout Mice

If the replacement gene ( $A^*$  in the diagram) is nonfunctional (a 'null' allele), mating of the heterozygous transgenic mice will produce a strain of 'knockout mice' homozygous for the nonfunctional gene (both copies of the gene at that locus have been knocked out).

Knockout mice are valuable tools for discovering the function(s) of genes for which mutant strains were not previously available. Two generalisations have emerged from examining knockout mice:

- Knockout mice are often surprisingly unaffected by their deficiency. Many genes turn out not to be indispensable. The mouse genome appears to have sufficient redundancy to compensate for a single missing pair of alleles.
- Most genes are pleiotropic. They are expressed in different tissues in different ways and at different times in development.

## Transgenic Sheep

Until recently, the transgenes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a transgene into a specific gene locus was reported. The gene was the human gene for  $\alpha$ 1-antitrypsin and two of the animals expressed large quantities of the human protein in their milk.

This is how it was done.

Sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA:

1. 2 regions homologous to the sheep COL1A1 gene. This gene encodes Type 1 collagen. (Its absence in humans causes the inherited disease osteogenesis imperfecta.)

This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.

2. A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector.
3. The human gene encoding alpha1-antitrypsin.

Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease Alpha1-Antitrypsin Deficiency (A1AD or Alpha1). The main symptoms are damage to the lungs (and sometimes to the liver).

4. Promoter sites from the beta-lactoglobulin gene. These promote hormone-driven gene expression in milk-producing cells.
5. Binding sites for ribosomes for efficient translation of the mRNAs.

Successfully transformed cells were then

- fused with enucleated sheep eggs and
- implanted in the uterus of an ewe (female sheep).
- Several embryos survived until their birth and two young lambs have now lived over a year.
- When treated with hormones, these two lambs secreted milk containing large amounts of alpha1-antitrypsin (650  $\mu\text{g/ml}$ , 50 times higher than previous results using random insertion of the transgene).

## Transgenic Chickens

### Chickens

- grow faster than sheep and large numbers can be grown in close quarters;
- synthesise several grams of protein in the 'white' of their eggs.

Two methods have succeeded in producing chickens carrying and expressing foreign genes.

- Infecting embryos with a viral vector carrying
- the human gene for a therapeutic protein
- promoter sequences that will respond to the signals for making proteins (e.g. lysozyme) in egg white.
- Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.

Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay.

Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins - something that *E. coli* cannot do.

## Transgenic Pigs

Transgenic pigs have also been produced by fertilising normal eggs with sperm cells that have incorporated foreign DNA. This procedure, called sperm-mediated gene transfer (SMGT) may someday be able to produce transgenic pigs that can serve as a source of transplanted organs for humans.

## Making Dolly

- Enucleate the eggs produced by Scottish Blackface ewes (female sheep).
- Treat the ewes with gonadotropin-releasing hormone (GnRH) to cause them to produce oocytes ready to be fertilised. Like

all mammals, these are arrested at metaphase of the second meiotic division (meiosis II).

- Plunge a micropipette into the egg over the polar body and suck out not only the polar body but the haploid pronucleus within the egg.
- Fuse each enucleated egg with a diploid cell growing in culture.
- Cells from the mammary gland of an adult Finn Dorset ewe (they have white faces) are grown in tissue culture.
- Five days before use, the nutrient level in the culture is reduced so that the cells stop dividing and enter G0 of the cell cycle.
- Donor cells and enucleated recipient cells are placed together in culture.
- The cultures are exposed to pulses of electricity to
  - cause their respective plasma membranes to fuse,
  - stimulate the resulting cell to begin mitosis (by mimicking the stimulus of fertilisation).
- Culture the cells until they have grown into a morula (solid mass of cells) or even into a blastocyst (6 days).
- Transfer several of these into the uterus of each (of 13, in this case) Scottish Blackface ewes (previously treated with GnRH to prepare them for implantation).

The result: one ewe gave birth (148 days later) to Dolly.

### What made Dolly different?

The Wilmut group also used the same technique to produce healthy lambs using cells from lamb embryos (9 days after fertilisation) and lamb foetuses (26 days after fertilisation). But in



these experiments, there was no way to know the phenotype of the nuclear donor because it had not yet been born. So, too, the recent cloning of monkeys from embryo nuclei represents simply an expansion of nature's ability to produce identical twins, etc. whose traits we will not know until they are born and grow up. But the nucleus that made Dolly came from an adult animal whose phenotypic traits were there to be seen.

### **How do we know that Dolly is not the progeny of an unsuspected mating of the foster mother?**

- She has a white face and the foster mother is a Scottish Blackface
- DNA fingerprinting reveals bands found in Finn Dorset sheep (the breed that supplied the mammary cells), not those of Scottish Blackface sheep

## **Cloning Cows**

Using somatic-cell nuclear transfer, a group of scientists in Worcester, Massachusetts reported that they had produced 6 cloned calves using nuclei from somatic cells that had been grown in culture until they were close to the end of their ability to proliferate.

Surprisingly, the cells of the 6 calves

- not only did not have the shortened telomeres found in the donor cells (and in Dolly), but had telomeres longer than those in normal newborn calves;
- could live in culture far longer than the ~50 cell doublings characteristic of cells from a normal newborn mammal.

## **Cloning Mice**

Wakayama, reported that they succeeded in cloning mice from fully differentiated adult cells. These workers used a nuclear injection technique similar to the one for frogs. Their success rate was 2-3%.

## Cloning Mules and Horses

On 4 May 2003, a horse gave birth to Idaho Gem, a healthy mule. It had developed from an enucleated horse oocyte that had received a nucleus from a cell of a mule foetus (produced by mating a male donkey with a female horse). Although widely reported as a clone, not yet! Sometime later in the year, two other mules derived from the same somatic-cell nuclear transfer procedure are expected to be born. These will be true clones of Idaho Gem.

And in August 2003, Italian scientists reported that they had cloned (by somatic-cell nuclear transfer) a baby horse. Because the donor nucleus had been taken from a cell of the mare that was implanted with the blastocyst, she gave birth to her identical twin!

Great interest is being shown in using the technique that produced Dolly (and Idaho Gem) ('somatic-cell nuclear transfer') to create embryonic stem cells that could be used to replace missing or defective cells in the body of the nuclear donor.

But the same blastocysts could, in theory, be instead being implanted in a uterus to produce a foetus that was a clone of the cell donor.

- In humans, nuclear genes take over earlier in development (at the 4-cell stage) than they do in cattle and sheep (8 cells). Perhaps there will not be enough time for the DNA from an adult human cell to be reprogrammed so that all its genome can once again be expressed.
- In monkeys (and presumably in humans), removal of the resident nucleus also removes essential components of the centrosome. Although insertion of the donor nucleus triggers mitosis, the spindles are defective and the resulting cells become aneuploid, that is, fail to receive a correct complement of chromosomes.

Despite these concerns, a group of Korean scientists has succeeded in producing cloned human blastocysts by somatic cell nuclear transfer (SCNT). They used these to create embryonic stem cells and made no attempt to implant them to create a baby.

## What are the Future Prospects for Transplantation?

Although organ transplants have helped thousands of people, much remains to be done. In particular, ways need to be found to

- increase the number of available organs (the need now far exceeds the supply)
- find more precise methods of immunosuppression in order to prevent rejection without the dangerous side effects of infection and cancer.

Both these problems may be helped by xeno-transplantation. Xenotransplantation is the use of organs from other animals. A number of attempts have been made to use hearts, livers and kidneys from such primates as chimpanzees and baboons — so far with limited success. One reason is that xenotransplants usually are attacked immediately by antibodies of the host resulting in hyperacute rejection. But perhaps the use of pigs as organ donors will be feasible.

- Their organs are about the right size for use in humans.
- They can be made transgenic for molecules that may circumvent
  - hyperacute rejection (by knocking out the genes responsible for cell-surface antigens that humans have preformed antibodies against);
  - the chronic, T-cell-mediated, rejection that plagues all allografts.
- They can be produced in the numbers needed.

However, pigs contain retroviruses (called PERV = porcine endogenous retrovirus) and there is fear that these might infect the human recipient (in much the same way that a primate retrovirus seems to have made the jump to humans in the form of HIV, the cause of AIDS).

Only a few transplants of pig tissue into humans have been done to date: skin grafts and grafts of pancreatic islets. A larger number of people have been temporarily hooked up to pig organs or 'bioreactors'

containing pig cells to provide support for their failing spleen, liver or kidneys.

Most of these recipients have been monitored for signs of infection by PERV and — even though PERV can infect human cells growing in culture — there is no evidence that any of these people exposed to pig tissue have become infected.

## The Influence of Technical Confluence

In 1997 ‘Dolly’ and ‘Polly’ have changed how we look at cloning and transgenesis in multi-celled animals. Dolly, of course, is the famous ‘cloned sheep’ announced early this year and ‘Polly’ is a transgenic sheep from the same research program.

Before ‘Dolly’ and ‘Polly,’ cloning animals and making transgenic livestock were lines of research that proceeded independently since the mid 80’s.

An early step in cloning in livestock was to split a developing calf embryo and generate two genetically identical calves from the two embryonic halves.

The first step in transgenesis as practiced by bacteriologists was to add a new gene to a cell in culture and observe the effect of that one added gene, also in culture.

The successful birth and development of ‘Dolly’ the sheep showed that researchers could start with cells taken from adult animals, not just with embryonic cells. After the original cells were collected from the adult sheep, these cells were grown and multiplied in cell culture before being used to generate an embryo.

Growing and multiplying in culture populations of these totipotent cellsóindividual cells capable of going through embryonic and foetal development to produce an adult animal is a powerful tool. Rather than manipulating one cell at a time, animal scientists are starting to be able to manipulate cell populations like the way bacteriologists do: adding genes to a population of cells and selecting for the particular rare cells that have incorporated the desired combination of genes.



Once that particular rare cell is found, it can be grown from a single cell to a complete animal. This means scientists can hope to study gene effects not just on cell physiology in a test tube, but also on growth and development into tissues organs and complete animals.

Comparing what could be done in terms of transgenic animals before Dolly and what can be done using the 'Dolly technology' helps in understanding the impact that the 'Dolly technology' will have in research with transgenic animals.

## **Don't Just Add a Gene, Adjust It and Exchange It**

The next step in research with transgenic livestock is site-specific insertion of genes using homologous recombination to study the effect of exchanging one version of a gene for another. Homologous in this case means that you replace a gene with a different gene for the same kind of function, located in the same place on the organism's genome.

Using homologous recombination is powerful because a researcher can make an inactive version of a gene and exchange it for an active version. But homologous recombination is to random insertion as a needle is to a haystack. The researcher is challenged to find that particular cell in which the newly donated gene trades places with a target gene already on a chromosome. The researcher needs to distinguish that rare cell from the cells, which have the more common random insertion of the newly donated gene anywhere in the genome.

Imagine the problems of homologous recombination have been solved, but 'Dolly Technology' is not available. Researchers could routinely use a donor gene to exchange or 'knock out' the corresponding gene in a sheep cell. If the cells grow as undifferentiated tissue in culture, researchers could study the effect of the target gene on cell physiology. But researchers wouldn't know what, if any, function the gene may have in development or in the physiology of the adult animal.

## **The State of Cloning and Homologous Recombination: 1997**

So far, the only animal in which scientists have a system for inserting a known donor gene into a known site using homologous recombination is the mouse. Putting in a new version of a gene and 'knocking out' the existing version of the gene is the basis of knock-out mice.

You can look at the term knock-out in two ways. The more general is 'one is put into place as the other is knocked out of place.' The version of the gene you put in might work but in a different way than the existing version of the gene it replaces. More commonly the donated gene is inactive and in this sense the addition of the donated gene not only 'knocks out' the working copy of the gene, it can also knock out the normal gene's function.

In livestock, researchers can insert a known gene but the insertion is random. With livestock, it has been possible to add a new gene to an embryonic stem cell and then grow an adult animal from the cell. But so far it has been impossible to insert a new, modified version of an existing gene, replacing a normal version in a cell and then generate an adult from that cell. A system of homologous recombination will allow researchers to study the effect of 'knocking-out' existing genes to study the genes functions.

This is why homologous recombination will be so valuable in research on the genetics of livestock animals. Compare that with transgenesis. Transgenesis merely requires the random insertion of a new gene. The new gene inserts anywhere. It does not exchange or knock out of place any other gene.

## **Homologous Recombination in Transgenic Animals: The Mouse Model**

The challenge of harnessing homologous recombination is to get not random insertion but rather a specific exchange, where the donor

gene is exchanged for and displaces the homologous existing resident gene.

When a researcher adds a donor gene to a population of cells, there are at least three possibilities with each cell.

1. The donor gene fails to insert.
2. The donor gene randomly inserts anywhere in the genome.
3. The donor gene by homologous recombination specifically inserts at the site of its homologous gene and displaces it.

This involves a significant technical challenge for the biologist: How to select for cells that have exchanged genes and select against cells that have received the donor gene through random insertion?

1. The donor gene is usually not easy to find when it inserts.
  - A. Consider attaching to the donor gene, a second gene that is easy to find.

If you can find those cells in which the two connected genes have inserted into chromosomes, you have found those cells that have the target donor gene. A common gene for resistance is *neo*. It confers resistance to an antibiotic called G 418. Keep in mind that the genes could be inserted randomly anywhere or they could be inserted by homologous recombination at the target site.

- B. Consider inserting the selectable *neo* marker into the donor gene, thereby inactivating the donor gene and yet it keeps its ability to undergo homologous recombination.
2. Consider attaching a third gene. Attach the third gene next to the donor gene. The third gene is next to but outside the region of homology. Consider the third gene to be a suicide gene or a counter-selectable marker that works opposite the way of a selectable marker. A suicide gene makes a cell vulnerable to a chemical or antibiotic. The standard example is the TK gene or thymidine kinase gene. Cells that have and express the TK gene are killed by the antibiotic Gancyclovir. Cells without TK gene are not harmed by the drug.

3. Here is the discerning part. If the donor DNA inserts randomly, then all three genes will be inserted: the inactive donor gene and the two active marker genes. That means the cell will both resist the first antibiotic (G 418) but will be killed by Gancyclovir.

On the other hand, if the donor DNA inserts by homologous recombination, then only the homologous stretch of DNA is inserted. The flanking TK gene is outside the region of homology and it is not inserted. Instead it is jettisoned. The neo gene inserted within the donor gene is inserted, because it is between the two regions of homologous DNA. That means that cells with homologous recombination resist both G 418 because they have neo and Gancyclovir because the cells lack TK gene.

This means if you start with a population of cells growing in cell culture, you can generate and select for and obtain cells that have, through homologous recombination, exchanged a donor gene for an existing gene.

Until today, this means you can study the effect of the donor gene, but only in cells grown in tissue culture. And only in the heterozygous condition. Since each cell usually has two copies of each gene, the donor gene is likely working in the presence of the second copy of the original gene, on the sister chromosome.

(Heterozygous in this discussion means that the gene of interest is different in each sister chromosome. Homozygous means it is the same on each sister chromosome.)

## Cloning: From Single Cell to Whole Animal

How can you go from having a bunch of single cells with single copies of the donor gene in the presence of another copy of the original homologous gene, to having complete animals that are homozygous for the donor gene?

1. You need to grow a complete animal from the single cell that has one copy of the donor gene. That animal is heterozygous for the gene you're studying.

2. You need to breed that animal with another heterozygous animal. This is basically the same as crossing two animals both heterozygous for the gene of interest.
3. You need to test the offspring. Each of the offspring or progeny of the cross has a 25 % chance of being homozygous for the donor gene of interest. Finding which offspring are homozygous is easiest if there is a DNA fingerprint test that discerns between the donor gene and the original gene. With such a test, each progeny can be assayed and those found to be homozygous for the donor are studied further.

### **How can a researcher grow a complete animal from a single cell, one that is not a zygote?**

Here is how it is done with pre-Dolly technology. Let's say you take an embryo at the 32-cell stage and disrupt it into 32 individual cells. If you grow it in the appropriate medium and conditions, the cells will divide in culture. You can grow hundreds of these cells. These are called 'embryonic stem cells.' One of their key traits is that they can develop into any type of tissue while in culture: muscle, nerve, heart and so forth.

You can also add a gene or two to embryonic stem cells. You can select for those cells that have undergone homologous recombination. You can take several of these modified embryonic stem cells and inject them into a second embryo, again at about the 32 or 64-cell state. You get an embryo with two genetically distinct types of cells: the prevalent original type and the few that have the extra gene. As the mixed or 'chimaeric' embryo grows, all the cells derived from the modified embryonic stem cells have the extra gene, all the cells derived from the other cells do not have the extra gene.

If the cells that have the extra gene develop only into somatic tissues and organs and not into germline tissue, then the resulting adult chimaeric animal will give only offspring carrying unmodified genes. On the other hand, if the germline tissue derives from the modified cells, then the germline cells will have the extra gene and half of the

gametes produced by the chimaeric animal will have the added gene. The drawback is you have to grow the animal to maturity to be able to test the sperm cells or ovaes to see if they are carrying the gene of interest.

Mating a heterozygous male and a heterozygous female is expected to result in 1/4 of the progeny to be homozygous. The progeny can serve as founders for homozygous lines of animals that have knocked out a particular gene.

## **How to Simplify it?**

### **Bypass the chimeric embryo using ‘Dolly technology.’**

Imagine if instead of starting with modified embryonic stem cells that have to be injected into a 32-cell embryo, you could instead take the modified embryonic stem cells and generate an adult animal from a single cell. That means all the cells of the embryo that develops from the original single cell will all have the copy of the gene. You would therefore bypass the chimaeric phase. Neither Dolly nor Polly would be chimeras.

You would still have to deal with a founder animal that is heterozygous. But this simplifies the system by reducing the need for one entire generation. Instead of chimaera to heterozygote to homozygote, you can go from heterozygote directly to homozygote. With mice, that is a savings of a few weeks. With livestock, that could mean a savings of 2 to 3 years.

If you can grow an adult sheep from cells taken from an adult and grown in test tubes, then you can take a population approach: grow many cells, genetically manipulate them as a group, select for those cells that have the desired

combination of traits. Take individual selected cells and from them grow an entire adult. Breed that adult with another heterozygous

adult and you have a 1/4 chance of getting progeny that are homozygous for the inserted gene.

We anticipate that the news media will be dumbfounded when a researcher announces the development of a system of homologous recombination. I think the general public will have a hard time grasping the significance. With the cloning of 'Dolly' came the possibility of cloning adult humans, a long-standing topic of science fiction books and movies. But as far as I know, nobody has made any movies about homologous recombination. It's important but sublime, even obtuse.

For geneticists, the sublime will be significant. From an applied point of view, modifying the genome of pigs by substituting the human version for the pig version of the major histocompatibility genes will 'humanise' pig organs, making them more likely candidates for transplanting to humans. From a fundamental point of view, modifying existing genes will speed our understanding of the roles of those genes in development from zygote to billy goat.

## Historical Background of Genetic Animals

Prior to the development of molecular genetics, the only way of studying the regulation and function of mammalian genes was through the observation of inherited characteristics or spontaneous mutations. Long before Mendel and any molecular genetic knowledge, selective breeding was a common practice among farmers for the enhancement of chosen traits, e.g., increased milk production.

During the 1970s, the first chimeric mice were produced. The cells of two different embryos of different strains were combined together at an early stage of development (eight cells) to form a single embryo that subsequently developed into a chimeric adult, exhibiting characteristics of each strain. The mutual contributions of developmental biology and genetic engineering permitted rapid development of the techniques for the creation of transgenic animals. DNA microinjection, the first technique to prove successful in mammals, was first applied to mice and then to various other species such as rats, rabbits, sheep, pigs, birds and fish. Two other main

techniques were then developed: those of retrovirus-mediated transgenesis and embryonic stem (ES) cell-mediated gene transfer.

Since 1981, when the term transgenic was first used by J.W. Gordon and F.H. Ruddle (1981), there has been rapid development in the use of genetically engineered animals as investigators have found an increasing number of applications for the technology.

## **Methods of Creation of Transgenic Animals**

For practical reasons, i.e., their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time and their fairly well defined genetics, mice have become the main species used in the field of transgenics.

The three principal methods used for the creation of transgenic animals are DNA microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer.

### **a) DNA microinjection**

This method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilised ovum. It is one of the first methods that proved to be effective in mammals. The introduced DNA may lead to the over- or under-expression of certain genes or to the expression of genes entirely new to the animal species. The insertion of DNA is, however, a random process and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. The manipulated fertilised ovum is transferred into the oviduct of a recipient female or foster mother that has been induced to act as a recipient by mating with a vasectomised male.

A major advantage of this method is its applicability to a wide variety of species.

### **b) Embryonic stem cell-mediated gene transfer.**



This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells. Stem cells are undifferentiated cells that have the potential to differentiate into any type of cell (somatic and germ cells) and therefore to give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development. The result is a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method.

This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

#### **c) Retrovirus-mediated gene transfer.**

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F1) of animals that need to be tested for the expression of the transgene. Depending on the technique used, the F1 generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for 10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

## **Transgenic Animals in Biotechnology**

Transgenic animals are just one in a series of developments in the area

of biotechnology. Biotechnology has transformed the way in which we understand processes such as engineering and manufacturing. These terms now include the use of living organisms or their parts to make or modify products, to change the characteristics of plants or animals or to develop micro-organisms for specific uses. The novel uses of biological techniques such as recombinant DNA techniques, cell fusion techniques, mono and polyclonal antibody technology and biological processes for commercial production have altered traditional distinctions and methods. Genetic manipulations at the level of DNA have also changed long held views as to what is considered to be animal, plant and human. In turn, these changes have made it more difficult to evaluate the ways in which animals are used and have obscured distinctions between pure and applied research.

Consideration of the acceptability of creating specific transgenic animal strains or genetic manipulation involving interchanging DNA between species and kingdoms could be a simple animal care issue or a societal decision. The following is an attempt to show what the ability to create transgenic animals or engage in other forms of DNA manipulation means in terms of traditional ACC functions, not forgetting that this impacts on wider considerations of human responsibility for the welfare of other life forms.

The creation of transgenic animals is resulting in a shift from the use of higher order species to lower order species and is also affecting the numbers of animals used. This shift in the patterns of animal use is being monitored by the CCAC through the use of the Animal Use Data Form.

An example of the replacement of higher species by lower species is the possibility to develop disease models in mice rather than using dogs or non-human primates.

In the long term, a reduction in the number of animals used, for example to study human diseases, is possible due to a greater specificity of the transgenic models developed. On the other hand, the success of the method has led to using its potential for investigating a wider range of diseases and conditions. The actual use of some species may

be increased, in addition to the numbers of animals, which are sacrificed as donors during the creation process. The potential of the technology has also made it possible to consider employing cattle, swine, sheep and goats as processing units to manufacture proteins or as organ donors.

The complex interactive processes of living mammals are not reproducible in vitro. However, transgenic animals provide a means of evaluating genetic modifications in terms of anatomical and physiological changes in a complex system. Transgenic models are more precise in comparison to traditional animal models, for example the oncomouse with its increased susceptibility to tumour development enables results for carcinogenicity studies to be obtained within a shorter time-frame, thus reducing the course of tumour development in experimentally affected animals. However, models are not strict equivalents, so as with any other system care must be taken in drawing conclusions from the data.

A representative, but non-inclusive, list of purposes for which transgenic animals have been used indicates the wide ranging application of this biotechnology:

- in medical research, transgenic animals are used to identify the functions of specific factors in complex homeostatic systems through over- or under-expression of a modified gene (the inserted transgene),
- in toxicology: as responsive test animals (detection of toxicants),
- in mammalian developmental genetics,
- in molecular biology, the analysis of the regulation of gene expression makes use of the evaluation of a specific genetic change at the level of the whole animal,
- in the pharmaceutical industry, targeted production of pharmaceutical proteins, drug production and product efficacy testing,
- In biotechnology: as producers of specific proteins,

- genetically engineered hormones to increase milk yield, meat production, genetic engineering of livestock and in aquaculture affecting modification of animal physiology and/or anatomy, cloning procedures to reproduce specific blood lines and
- developing animals specially created for use in xenografting.

Important general considerations include the extent to which experience acquired in the laboratory with regard to husbandry should influence industry standards for keeping animals created specifically as living machines for the production of proteins, antibodies, etc. What words are appropriate to describe and evaluate the condition of animals now used as production units? The successful cloning of Dolly underlines the fact that innovative developments in animal science are part of the mainstream of biotechnology. In addition, the use of xenografts, at least at the public health level makes animal and human welfare inseparable.

## **In Vitro Fertilisation and Embryo Transfer**

More than one million children have been conceived since the successful use of the IVF-ET technology. IVF is a procedure designed to enhance the likelihood of conception in couples for which other fertility therapies have been unsuccessful or are not possible. It is a complex process and involves multiple steps resulting in the insemination and fertilisation of oocytes (eggs) in our laboratory. The embryos created in this process are then placed into the uterus for potential implantation.

Each stage of the procedure is associated with specific risks, as outlined below.

### **Benefits**

It is an elective medical treatment. The therapy provides hope to a couple who has been otherwise unable to conceive with a chance to establish a pregnancy.

### **Risks of Superovulation Stimulates Egg Development**

The controlled 'superovulation' techniques used in IVF is designed to stimulate the ovaries to produce several eggs (oocytes) rather than the usual single egg as in a natural cycle. Multiple eggs increase the

potential availability of multiple embryos (fertilised eggs) for transfer and ultimately increase the probability of conception.

The medications required to boost egg production may include, but are not limited to the following:

- Lupron (gonadotropin releasing hormone-agonist)
- Antagon or Cetrotide (gonadotropin releasing hormone-antagonist)
- Follistim, Bravelle or Gonal-F (FSH, follicle stimulating hormone)
- Repronex (combination of FSH and LH, luteinizing hormone)
- Pregnyl, Profasi or Novarel (hCG, human chorionic gonadotropin)

Each is administered by injection only. Most medications are given subcutaneously (beneath the skin), though some are intramuscular injections (into the muscle). Risks associated with injectable fertility medications may include but are not limited to, tenderness, infection, hematoma and swelling or bruising at the injection site.

Risks associated with the medications may include, but are not limited to, allergic reactions, hyperstimulation of the ovaries (mild, moderate or severe), failure of the ovaries to respond and cancellation of the treatment cycle.

There are situations that can occur during a stimulation that may necessitate canceling your IVF cycle and stopping treatment for a period of time. This occurs because the ovaries produce either too many or too few eggs in response to drug stimulation protocol. Although we realise that this can be a big disappointment, at times it is necessary to discontinue the use of the medications to avoid the possibility of complications and to afford you the best chance of future success. If canceling the cycle becomes necessary, you will be told to stop your injections. No hCG injection will be given and no egg retrieval will occur. You will be asked to schedule an appointment with your physician to make decisions regarding future treatment cycles.

When ovulation induction medications are used in fertility therapy, the ovaries are coaxed to produce more than one egg to the point of maturity. Consequently, hormone levels of estrogen and progesterone reach much higher than normal values. When the estrogen level becomes mildly to moderately elevated, side effects that may be experienced include, but are not limited to, fluid retention with slight transient weight gain, nausea, diarrhoea, pelvic discomfort due to enlarged cystic ovaries, breast tenderness, mood swings, headache and fatigue.

## **Ovarian Hyperstimulation Syndrome (OHSS)**

If the estrogen level rises excessively and hCG is administered to trigger final maturation of the eggs, the following more serious complications may result:

1. Excessive fluid retention with fluid in the abdomen and/or chest cavity,
2. Thrombosis of arteries and/or veins (formation of blood clots) which may lead to stroke, embolus or potentially fatal complications,
3. Abnormally enlarged ovaries, which have the possibility of rupturing or twisting (a surgical emergency)

Any of the three problems listed above may require prolonged hospitalisation.

Given the potential for such severe complications, it is important that we carefully monitor your response to these medications. This monitoring also allows your physician to determine when the eggs are ready for the next stage, oocyte (egg) retrieval. Monitoring includes frequent blood drawing for estradiol (estrogen) and possibly progesterone, LH and FSH levels. These blood tests will take place over approximately a twelve-day period. Risks associated with blood drawing may include, but are not limited to:

1. Pain at the site of needle stick
2. Tenderness or infection of the skin

3. Bruising or scarring of the site of blood draw
4. Development of a blood clot in the vein (thrombosis, thrombophlebitis)

The second portion of the monitoring phase in IVF involves the use of intravaginal ultrasound to track follicular growth. The eggs develop inside fluid-filled cysts of the ovaries called follicles, which enlarge as the eggs mature. Ultrasound studies usually begin after an estrogen response has been measured and continue on a frequent basis until oocyte (egg) retrieval. The ultrasound studies are performed using a vaginal probe. Vaginal sonograms carry no appreciable risk but may cause slight discomfort, particularly as you near the point of ovulation.

Ovarian stimulation with the fertility medications causes multiple follicles to develop. This is desirable in IVF because as the number of eggs increases, the chance for success increases. Multiple embryos can also increase the risk of multiple pregnancies. Approximately 20-25% of pregnancies with IVF will be multiple. Most of these will be twins, but triplets, quadruplets or even greater multiple pregnancies can occur. A procedure called 'selective reduction of pregnancy' has been performed in several medical centres across the country in selected cases of triplets or more. Selective reduction is not offered on site or by GRS staff. More information on this procedure and recommended centres is available on request.

## Egg Retrieval

For IVF, collection of eggs is usually performed under transvaginal ultrasound guidance. To accomplish this, a needle is inserted (under IV sedation) through the vaginal wall into the ovaries using ultrasound to locate each follicle. The follicular fluid is drawn up into a test tube to obtain the eggs. Although patients are given pain medications intravenously and are carefully monitored by an anaesthesiology staff, some women may experience some discomfort during the procedure. Generally, the oocyte (egg) retrieval takes 20-30 minutes. Patients are usually discharged home within hours after the retrieval.



Risks of oocyte (egg) retrieval may include, but are not limited to, the following:

1. Potential reactions from the drugs and procedures used in the administration of anesthesia
2. Risks associated with the passage of the needle through the vagina into the ovaries (including infection, bleeding, inadvertent damage to adjacent structures including, but not limited to, the bowel, bladder, blood vessels, ureter, uterus or ovary(ies) and adhesion formation (internal scarring) following the procedure. Although uncommon, significant bleeding or damage to the bowel may occur and surgery may be required to repair such damage; this is a very uncommon event. Rarely, infection may become severe enough to require hysterectomy and/or removal of one or both ovaries.

## **The Sperm Collecting and Preparing**

By masturbation on the day of the oocyte (egg) retrieval, semen sample is obtained from the partner. This is usually obtained while the retrieval is being performed. Abstinence from ejaculation for two to five days prior to providing this semen specimen is recommended. After the specimen is produced, the sperm will be prepared for inseminating the collected eggs in the laboratory. Because this can be a stressful time period for men, the man/partner may be unable to produce a specimen when needed. Men who feel that they may have difficulty producing a semen specimen, have the opportunity to have their specimens frozen by the laboratory ahead of time for use in this situation. Testicular biopsy can also be performed as a method to extract sperm for IVF.

## **Embryo Culture and Insemination**

After the retrieval of the egg follicular fluid is immediately transferred to the adjacent laboratory for identification of eggs, evaluation and preparation for insemination. In the process of collecting the follicular fluid, it is possible that a large number of eggs may be retrieved. It is

strongly recommended that all of these eggs be inseminated to maximise the number of embryos available for subsequent transfer. Any objection(s) to this policy should be stated in writing and attached to the IVF-ET consent form with the understanding that pregnancy success may be reduced. Otherwise, the prepared sperm will be added to each egg and they will be allowed to incubate overnight under controlled laboratory conditions. The next day, each egg is evaluated for evidence of fertilisation. However, it is possible that no eggs are fertilised. If this happens, the laboratory staff will re-inseminate the eggs or perform intracytoplasmic sperm injection (ICSI) in the hope of obtaining embryos for transfer. If fertilisation still does not occur, the eggs will be discarded and the remainder of the procedure will be cancelled. In the case of severe male factor, the couple may be asked to consider the option of using anonymous donor sperm (obtained through a licensed sperm bank for use as a “backup” or secondary sperm source) if it is not possible to obtain sufficient sperm from the partner at the time of fertilisation.

The eggs that have fertilised will be allowed to develop for two or more additional days under controlled laboratory conditions before they are placed inside the woman’s uterus. Depending upon the couple’s wishes, some fertilised eggs/ embryos may be frozen and stored for future use.

After the embryos are transferred to the womb, the woman will continue progesterone supplementation that begins on the evening of the egg retrieval procedure. Progesterone can be taken as a combination of oral troches and rectal/vaginal suppositories or by injections. Administration of these medications after egg collection has been shown to create a more favourable uterine environment for the embryos, which therefore increases pregnancy rates.

Side effects of progesterone may include, but are not limited to the following:

1. Vaginal dryness;
2. Bloating, breast tenderness;

3. Depression, mood swings;
4. Delay of menses.

Synthetic progesterone-like medications have been associated with certain birth defects. By using only natural progesterone, the risk of drug-induced birth defects is significantly reduced. It is important to note, however, that birth defects occur in approximately 3% of spontaneously-conceived pregnancies in the USA. Therefore, use of natural progesterone does not guarantee a child without a birth defect.

## **Embryos Transfer to the Uterus**

Embryos are transferred on either day three or day five of development. The embryologists at GRS are highly skilled in identifying 'healthy' embryos and in some cases will recommend that a patient extend embryo development to day five, known as the blastocyst stage. Blastocyst transfer has become quite common in IVF cycles as it can increase chances for success while decreasing the likelihood of multiples. The physician will work closely with the embryologists to determine if day three or day five transfer would be ideal for your cycle.

Embryos are transferred to the uterus through a small tube (catheter). This procedure is much like a pap smear and does not require any anesthesia and is usually painless. The embryos are placed in a small amount of fluid inside the catheter, which is passed through the cervix at the time of a speculum examination. The embryos are placed in a manner so they reach the top part of the uterus. The number of embryos transferred depends on individual circumstances of the couple and this decision will be made collectively by the patient, the physicians and the embryologist. Typically, two to four embryos are transferred in one treatment cycle.

Embryo transfer can cause mild cramping. Although unlikely, during the embryo transfer the embryo(s) may be displaced through the cervix (causing loss of embryos) or into the fallopian tubes (causing possible tubal pregnancy). There is a small risk of bleeding or infection as a result of the transfer procedure.

After transfer, the woman may get dressed and leave after a brief recovery period. A pregnancy test will be done twelve to fourteen days after the transfer, regardless of the occurrence of any uterine bleeding.

The transfer of several embryos increases the probability of success. A multiple embryo transfer also increases the risk of a multiple pregnancy. Any multiple pregnancies carry an increased risk of miscarriage(s), premature labour and premature birth as well as an increased financial and emotional cost. Pregnancy-induced high blood pressure and diabetes are more common in women pregnant with more than one foetus. Prolonged hospitalisation may be necessary for these pregnant women and for the mother and babies after delivery. Tubal (ectopic) pregnancy is also possible and a combination of normal pregnancy and ectopic pregnancy may occur. A tubal pregnancy is a condition that may require laparoscopy or major surgery for treatment. Like spontaneous (natural) conceptions, pregnancies that arise through IVF may result in miscarriage. In the event of a miscarriage, a dilatation and curettage (D&C) may be necessary.

Couples going through therapy must choose and formalise their choice in the appropriate GRS consent form by indicating one of the following options for handling of any remaining embryos:

1. Freezing (cryopreservation) of remaining embryos for use by the couple in future treatment cycles
2. Anonymously donating the embryos for use by another infertile couple(s), if the donating couple and the donated embryos meet the screening criteria (You will not receive any money for this donation, nor will GRS 'sell' them. GRS reserves the right to cryopreserve (freeze) any donated embryos as well as the right to discard any donated embryos if a suitable woman cannot be identified to receive the embryos)
3. Allowing the embryos to develop in the laboratory until they perish, at which time they would be disposed of in a manner consistent with professional ethical standards and applicable legal requirements (This usually occurs within six to eight days after egg collection)

## Other Issues:

Any assisted reproduction process or technique can be psychologically stressful. Significant anxiety and disappointment may occur. We encourage you to consider short-term supportive counselling during this time and we are happy to provide you with a list of psychiatrists, psychologists, counsellors and social workers who may help you through this difficult time.

A substantial time commitment is required by both partners to complete an entire course of IVF therapy. It will be necessary for couples to adjust their schedules to undergo the required testing and therapies associated with IVF-ET. It is the responsibility of the woman to report to our office as scheduled for repeated ultrasound examinations and blood tests over several days or weeks before and after the expected time of egg collection. It is the responsibility of the man to be available at the time identified by the physician to provide sperm.

## Concern and Potential Success of the Therapy

Unfortunately, neither conception nor a successful outcome of pregnancy is guaranteed by the IVF-ET procedure. There are many reasons why pregnancy may not occur with the IVF-ET procedure. In fact, there are complex and largely unknown factors that limit pregnancy rates following assisted reproductive techniques. Some of the known reasons for failure may include, but are not limited to:

There may be a failure to recover an egg because follicles that contain mature eggs may not develop in the treatment cycle.

- Follicles that contain mature eggs may not develop in the treatment cycle.
- Ovulation has occurred before the time of egg recovery
- One or more eggs cannot be recovered
- The eggs that are recovered may not be normal,

- There may be insufficient semen to attempt fertilisation of the recovered eggs because the man is unable to produce a semen specimen, because the specimen contains an insufficient number of sperm to attempt fertilisation, because the laboratory is unable to adequately process the specimen provided or because the option to use a donor sperm as a 'backup' was declined,
- Fertilisation of the eggs to form embryos may fail even when the egg(s) and sperm are normal,
- The embryos may not develop normally or may not develop at all. Embryos that display any abnormal development will not be transferred,
- Embryo transfer into the uterus may be difficult/impossible or implantation(s) may not occur after transfer or the embryo(s) may not grow or develop normally after implantation,
- Any step in the IVF-ET process may be complicated by unforeseen events, such as hazardous or catastrophic weather, equipment failure, laboratory conditions, infection, human error and the like.

In the event the couple should die before embryo transfer, the embryo(s) will be discarded unless other provisions are made in writing.

When pregnancy occurs following IVF, it will typically be a normal pregnancy. However, there is always a risk of abnormal pregnancy, miscarriage, blighted ovum, ectopic pregnancy or premature delivery. This is because the process of IVF-ET does not protect against such normal occurrences. Congenital abnormalities, genetic abnormalities, mental retardation or other birth defects which occur in approximately 3% of spontaneously-conceived pregnancies may still occur in children born following assisted reproductive techniques. A large review of a subset of children born following assisted reproductive procedures found the incidence of developmental anomalies similar to a control group of children spontaneously conceived. Women with multiple pregnancies have a much higher risk of complicated pregnancies, which may include the following:

toxemia, pre-eclampsia, miscarriage, premature labour and delivery, stillbirth, birth defects and other complications.

## **Different Alternatives to the IVF-ET Therapy**

Depending upon the individual and unique causes of infertility for each couple, the chance of conception through alternative means, including intrauterine insemination (IUI) and medicinal therapy, other than IVF-ET may or may not exist. Possible success rates of these alternatives may vary depending upon the type and severity of the cause of the infertility. For some couples, it may even be possible to conceive spontaneously without a physician's help. We should discuss these alternative treatment methods with our physician before we proceed with IVF-ET therapy.

## **Micromanipulation**

Since the birth of the first baby achieved through conception outside of the human body in 1978, the principles of 'in vitro' (literally 'in glass') fertilisation and culture have remained the same - careful establishment and maintenance of a well-controlled, sterile environment in which the normal physiology of fertilisation and early development can be played out relatively undisturbed to provide healthy embryos for transfer back into the body. During the ensuing two decades, much has been learned, however, about the tolerances of such a system and how this technique can be exploited to treat a widening range of infertility cases. There have been great strides made in development of more appropriate culture media that has enabled embryos to be grown for extended periods of time in culture. Surplus embryos and possibly eggs may now routinely be cryopreserved in liquid nitrogen for use in subsequent attempts at pregnancy. Fertilisation itself is no longer a hit-and-miss affair with the advent of assisted fertilisation through micromanipulation. Embryos can be micro-manipulated for cell biopsy to determine their genetic status as well as aid in their ability to implant through drilling into their outer shell (assisted hatching).

## Conventions in vitro fertilisation (IVF)

Through the controlled application of ovarian hyperstimulation, it is current practice to time the retrieval of mature oocytes (eggs) from a woman's ovary. The yield may vary anywhere from one to 30 or more eggs that may be retrieved depending on the responsiveness of the ovaries to the gonadotropins used to stimulate them. These eggs are gathered by the embryologist into an appropriately balanced salt solution and maintained at body temperature (37°C) until such time as they are ready to be inseminated. Meanwhile, a sample of semen containing the sperm destined to be used for each specific set of eggs is collected and processed by cell separation techniques to provide as clean and active a sample of sperm (atozoa) as possible. A major emphasis of the IVF laboratory is directed toward guaranteeing that the correct sperm go with the right eggs through good labelling and check systems. Ultimately, following several hours in culture, eggs and sperm can be mixed and allowed to bind and fertilise in a relatively natural fashion. Depending on the quality and maturity of both eggs and sperm, it is common for fertilisation rates to vary considerably relative to the original number of eggs collected. Twenty eggs retrieved in no way guarantees 20 embryos. Likewise, 20 fertilised eggs in no way guarantees that there will be 20 embryos of sufficient quality for both cryopreservation and fresh transfer to the woman's body.

Central to the question of how many embryos are actually utilised in any IVF treatment cycle is the period during which the embryos are cultured in vitro. This can be as little as one day or up to seven in the case of blastocyst growth and transfer. Assuming that culture conditions are relatively optimal, there is less and less reason not to culture embryos throughout their pre-implantation stages to allow the embryos to 'select' themselves for transfer or cryopreservation. The blastocyst is the term given to the very last stage of an embryo prior to it implanting into the endometrial lining of the uterus. The poorer the rates of blastocyst growth are, the more restricted the choice of embryo is at this stage of development. In any event, growth of any embryos to the blastocyst stage improves the level of discrimination of embryo viability available to the embryologist and is key to reducing the



numbers of embryos used for uterine transfer. The more confidence a clinic has in the viability of the embryos it transfers, the less need there is for multiple transfers of three or more embryos. Thus with the transfer of three or less embryos, the risk of multiple pregnancies is significantly reduced, in turn minimising risks of pregnancy loss or foetal abnormalities common in multi-foetal pregnancies.

## **Micromanipulation in IVF Therapy**

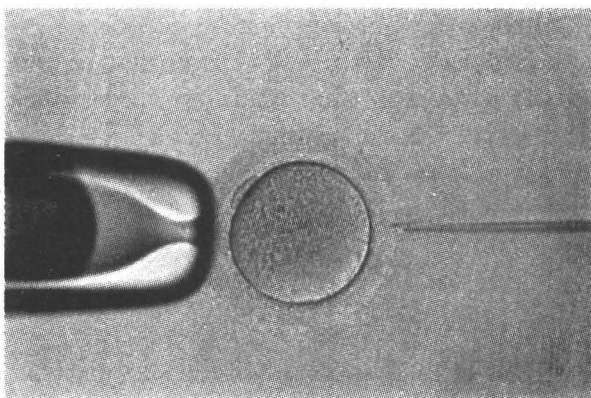
Micromanipulation is the technique whereby sperm, eggs and embryos can be handled on an inverted microscope stage, performing minute procedures at the microscopic level via joysticks that hydraulically operate glass microtools.

## **Male Factor Infertility**

Micromanipulation first saw clinical use in IVF for purposes of assisted fertilisation in the treatment of male factor infertility, where fertilisation potential was low in cases of poor sperm quality. The ultimate evolution of this approach has been the development of the single sperm injection procedure referred to as Intracytoplasmic Sperm Injection or ICSI. Sperm of virtually any quality and from any level of the male reproductive tract may be used with the only criterion for use being that the sperm is alive even if it is not moving (motile). Dead sperm may be able to achieve fertilisation, however, the DNA or genetic material from such sperm is too degenerate to form a viable embryo. Immature sperm from the testicle or the epididymis can be retrieved for use with ICSI for men who possess no sperm in their ejaculated semen (azoospermia). This azoospermia is either due to an obstruction in the tract (obstructive) or to extremely low production of sperm in the testicle itself (non-obstructive). In certain cases, men may produce sufficient sperm, but they do not survive to the point of ejaculation (necrozoospermia). Consequently, instead of using non-viable sperm from the ejaculate, testicular biopsy will provide a ready source of freshly produced viable sperm.

## **Intracytoplasmic Sperm Injection (ICSI)**

With the almost unlimited potential to achieve some level of fertilisation with ICSI regardless of sperm quality, it would seem that male factor infertility would no longer be of concern. It must be



noted, however, that sub-fertility in men can be related to certain numerical and structural defects of the chromosomes and, therefore, there is a strong recommendation for all couples that achieve pregnancies from ICSI to undergo prenatal screening. In certain cases of obstructive azoospermia, there is a higher incidence of cystic fibrosis in the male. Hence, before embarking upon treatment of the more extreme forms of male factor infertility, it is advisable to have some cytogenetic screening performed. Incidentally, very subtle compromise in sperm quality may well be responsible for a marginally lower embryonic viability rate and a slightly higher early miscarriage rate even if such embryos implant. Such observations have led to the suggestion that the technique ICSI itself is at fault, but this misses the point that ICSI per se is not causing the problem, merely facilitating the use of sperm, which under other circumstances would never have even achieved fertilisation.

## **ICSI for Non-Male Factor Infertility**

The use of ICSI is now routinely applied to a range of clinical situations wherever there is a possibility that conventional in vitro fertilisation may be suppressed or not occurs. Such situations include the following: idiopathic or unexplained fertility, hyper-responsive ovarian

stimulation cases where egg quality may be reduced, post-thaw sperm samples that survive poorly, post-thaw egg insemination, generation of embryos for pre-implantation genetic screening where embryos 'clean' from any extraneous contaminating sperm is needed or, indeed, any case where there is an extreme need to maximise normal fertilisation, for example, when a woman has only a few eggs retrieved. It is possible to 'rescue' cases following complete failed conventional fertilisation with ICSI.

The viability potential of these 'late-fertilised' embryos is



approximately half of timely fertilised embryos; nevertheless, they do generate successful live births. ICSI has become such a common feature of IVF therapy that it is fast becoming the insemination technique of choice.

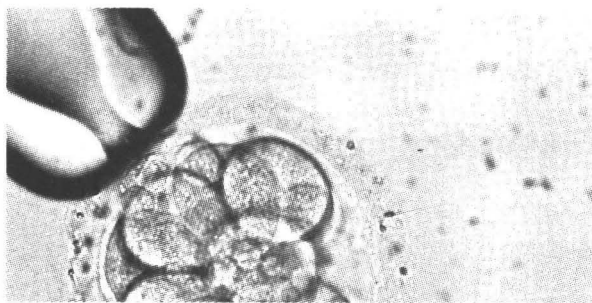
## Assisted Hatching

It has been proposed that a certain number of otherwise viable embryos do not implant simply because they are unable to break free from the surrounding 'jelly coat' (zona pellucida) when they reach the blastocyst stage of development. Around an unfertilised egg there exists a transparent glyco-protein coat that acts to protect the egg and regulate normal fertilisation by any penetrating sperm. This jelly-like coat continues to protect the early preimplantation embryo until, as a blastocyst, the embryo fills itself up with fluid like a water-filled balloon, pumping itself larger and larger until it ruptures and 'hatches' from the zona pellucida. The embryo is now ready to make contact in its naked form with the endometrium and implant. Inappropriate ovarian environment due to advanced maternal age or other factors that may compromise the follicular environment may in certain cases render the zona pellucida thicker or tougher. Such IVF cases may benefit from the application of a form of micromanipulation referred to as 'assisted hatching' In this process, the embryo has a hole made in the surrounding zona pellucida prior to transfer to enable it to 'hatch'

free from the zona pellucida more easily when it expands as a blastocyst in the uterus. This technique has never been unconditionally proven to be effective in any well-defined group of IVF patients and as such remains essentially an experimental procedure. Holes in the zona pellucida may be made mechanically, chemically or by laser. With the advent of more routine transfer of blastocyst stage embryos, the future of this technique, usually carried out on day three of development, may seem in question. Indeed, at the blastocyst stage in vitro, it may be most appropriate to dissolve off the entire zona pellucida prior to transferring naked embryos into the uterus. This could be considered the ultimate form of assisted hatching without the need for micromanipulation. Currently, however, assisted hatching can be easily performed using an infrared laser to create a hole in the zona pellucida that allows the embryo an easy means of escape when it is time to try and implant into the uterine wall.

## Embryo Biopsy

Briefly, it is of relevance in any discussion of micromanipulation techniques to mention the potential to biopsy both eggs and embryos. This



approach is known as preimplantation genetic diagnosis (PGD) and enables the screening of both the unfertilised egg by removal of the first polar body or the fertilised multi-cellular embryo by removal of one or more cells either at the 6-12 cell stage or from the trophectoderm of the blastocyst. This material can be probed for both genetic mutations or gross chromosomal errors. This technology remains in its infancy and can be of profound importance clinically, but at this time only for cases with very clear medically-defined needs. The biopsy procedure requires very exacting skills of the IVF laboratory

and the egg or embryo is not entirely free of risk during the procedure. Hence, couples whose offspring have a high chance of inheriting a genetic disorder may have their embryos screened. Women who are at risk of generating eggs with a high risk of chromosomal anomalies can benefit from having their eggs or embryos screened for chromosomal normality. While embryos can have their sex determined through this procedure, the GRS team considers it inappropriate to do so except in cases of sex chromosome-linked disorders.

## Conclusion

It is a privilege for any scientist or clinician to have access to the earliest stages of human development through culturing gametes and embryos in vitro. And, as such, it requires a high degree of ethical responsibility to provide as safe and optimal an environment as possible for these microscopic changes. While much has been done to maximise IVF pregnancy rates over the last two decades, it nevertheless remains to improve individual embryo selection to the point where we can routinely transfer only one embryo at a time, while being able to successfully and consistently freeze all surplus embryos of sufficient quality for later use in attempting pregnancy.

## Microinsemination

Microinsemination is a procedure that can be used to increase the chance of fertilisation for a couple undergoing in vitro fertilisation and embryo transfer (IVF-ET) who may have a reduced chance of fertilisation through standard egg insemination procedures. Clinical situations in which the techniques of assisted fertilisation may be useful include cases of male infertility, immunological infertility or when there has been failure of fertilisation or low rate of fertilisation in previous IVF treatment sessions. The method of microinsemination used at GRS is intracytoplasmic sperm injection or ICSI.

Eggs and sperm are obtained by using standard methods during an in vitro fertilisation treatment cycle. Sperm are then prepared in a manner to select and retain only the most active sperm in a

small volume of culture medium. After exposing the mature eggs to an enzyme that removes the cumulus cells which surround the egg, each egg is placed under a microscope and held in place by gentle vacuum with a small glass tube called a micropipette. A single sperm is then drawn up into an extremely sharp, hollow glass needle along with a very small amount of the nutrient liquid medium. The needle is then passed through the zona pellucida (the gel like substance surrounding each egg) and the cell membrane to inject the sperm into the centre of the egg by using a special microscope assembly. Approximately 16-18 hours after ICSI, the eggs are examined under the microscope to assess for the presence of two distinct pronuclei, which indicates normal fertilisation. Subsequent maintenance of embryos and the performance of the embryo transfer is the same as standard IVF.

## **Risks of Microinsemination**

There are many thousands of on going pregnancies and babies born worldwide since the introduction of ICSI. Unforeseen technical problems may arise which preclude successful fertilisation via microinsemination. The likelihood of success cannot be predicted.

## **Benefits of Microinsemination**

Microinsemination may increase the chances of pregnancy in couples whose chance of successful fertilisation through standard IVF techniques is reduced. While microinsemination may increase the chances of becoming pregnant, there are no assurances, either stated or implied, that microinsemination may result in pregnancy.

The chance of any woman giving birth to a child with congenital birth defects in the United States is 3-4 %, no matter how pregnancy is achieved. While available data does not indicate any reason to expect that microinsemination will result in increased incidence of chromosomal abnormalities in human infants.

## **Alternatives To Microinsemination**

The alternatives to microinsemination include increasing the sperm numbers that surround the egg while it is incubating in the laboratory or, in cases of male factor infertility, the use of donor sperm. Increasing the sperm concentration may increase the chance of fertilisation but may have an adverse effect on the laboratory environment of the egg.

## **The Relation Between the Genes and the Diseases**

The genetic disorders are organised by the parts of the body that they affect. Currently over 80 genetic disorders have been summarised and the content of genes and diseases are continually growing.

A subtle damage to the DNA is the cause of genetic or hereditary diseases. These diseases pass from generation to generation. The damaged gene is inherited according to the rules of genetics, although the pattern of inheritance may not be obvious without detailed scientific study. The majority of genetic disorders afflicting pedigree dogs show a recessive pattern of inheritance. In these conditions, two copies of the damaged gene, one inherited from the sire and the other from the dam, must be present for an individual to suffer from the disease. Carriers, which have only one copy of the damaged gene, will show no symptoms but can transmit the gene to successive generations. On average, when two such carriers are mated, 25% of the offspring will be affected with the disease - but another 50% will be carriers able to pass the disease on!

A dog can live with certain things that do not affect its health. In some instances we are not really dealing with a disease but a defect like malocclusions (poor tooth alignment), colouration etc. Though important in maintaining the breed within the standard set by the parent clubs and these should not be dismissed, they should be kept in perspective of the severity of other genetic disease and defects; those that cause severe health problems and even death.



We have DNA tests to examine directly the genetic code of the damaged gene. This can clearly differentiate normal, affected and carrier individuals. The information in the DNA does not change during the course of a dog's lifetime, so the tests, which require only a small sample of blood or a swab of cheek cells, can be done on a puppy. DNA tests detecting disease mutations are now available for a number of conditions including, globoid cell leukodystrophy in Cairns & Westies, copper toxicosis in Bedlington Terriers, vonWillebrands disease in Scottish Terriers, progressive retinal atrophy (PRA) in Irish Setters and fucosidosis in English Springer Spaniels. Research groups around the world are busy searching for mutations causing a number of other important hereditary diseases. Most conditions are breed specific, similar diseases in other breeds are not necessarily caused by the same mutation in the same gene. For example, in the case of PRA the disease in Irish Setters is unique to that breed and is a different condition from PRA in the Tibetan Terrier or in the Miniature Longhaired Dachshund. One of the few exceptions is PRA in Labradors (GPRA) and Miniature Poodles, which appear to be genetically similar. Finding the one gene causing a particular disease could be an intimidating task.

However, there are several ways to narrow down the search. Research groups, are developing resources for canine genetics which will be useful in directing research towards the most likely genes for a given disease. The objective is to create a genetic map enabling them to concentrate the search for a disease gene to a particular segment of DNA. When complete, the map will consist of 250-300 anonymous DNA markers called microsatellites. Researchers anticipate that such a low-resolution map will be complete sometime next year. How will this help in the search? By comparing the inheritance patterns of each of the microsatellite markers with that of the disease, then they can identify the whereabouts of the disease gene on the DNA without knowing anything else about the gene. Immediately they have reduced the search to about 0.3% of the total DNA containing only some 150 of the 50,000 genes estimated to be in the dog. At this stage, the DNA microsatellite marker itself can provide a useful guide to whether a dog is a carrier, particularly if other members of the family can be

tested too. DNA testing for copper toxicosis in Bedlington Terriers is currently based on such a marker - the disease gene itself has yet to be identified for this condition. Once a DNA microsatellite marker for a disease has been found, then researchers can concentrate on identifying the mutated gene itself. To further reduce the number of candidate genes they can use their knowledge of the clinical symptoms. For example, if a disease affects the eye, we know that the gene involved has to be active in the eye. The list of mutations known to cause genetic diseases in man is growing all the time and can provide a guide to possible mutation sites in similar canine disorders. For instance retinal degeneration in man has been shown to be caused by 12 different genes. By putting together all this information, we can home in the most likely candidate genes and scan those for the mutation causing the disorder.

To set up a genetic study to track down a disease gene using DNA markers, researchers need blood samples from families with both clinically normal and clinically affected dogs. They need a minimum of 50 to 60 samples but the more samples they have to test, the more precise the analysis. Dr. Nigel Holmes in his research for Tibetan Terrier PRA has used computer simulations to confirm that he now has a large enough number of samples and is beginning to screen them with the DNA microsatellite markers currently available. There is no way to predict which DNA microsatellite marker will be useful for a particular disease, it may be the first to be tried or it may only be found after several hundred markers have been tested.

Once we have a complete genetic map, however, we can be sure that there is a marker within that set that is in the vicinity of the disease gene that we are looking for and can carry out the search in a more methodical way. For analysis to be successful all the information on dogs that have been sampled must be 100% accurate, it only needs for one dog to be misdiagnosed to produce a false result from the genetic analysis. To ensure that this does not happen, researchers routinely establish criteria for the various conditions that they study to make sure that they can have total confidence in the diagnoses. Because the symptoms of progressive retinal atrophy become apparent at an early age and because the clinical signs are unambiguous, the assignment of disease status to family members

for this disorder has not been a problem. Lens luxation, however, is less straightforward. Perhaps ironically, it is the assignment of normal status, rather than affected status, that gives us problems in primary lens luxation. Although the first clinical signs can appear early, at 2 years or so, in some individuals the signs may appear as late as 8 years of age. The primary lesion is in a ligament attaching the eye lens, but the problem may only be noticed after the lens has shifted position. To ensure that none of the dogs identified to us as being clinically normal are in fact undiagnosed affected, we are now examining the information provided by breeders for each normal dog to confirm that they have all been diagnosed by a veterinary ophthalmology specialist after reaching the age of 9.

The development of canine genetics is at a very exciting stage. Over the next few years, the mutations underlying a number of important hereditary disorders will be identified and DNA tests devised. As our knowledge of canine genetics increases, progress in studying genetic diseases will accelerate. The availability of widespread DNA testing will give breeders the information to eliminate disease genes from their stock, whilst retaining genetic characteristics valuable to the breed. With DNA testing, carriers can be used with confidence since normal offspring can readily be identified. Originally DNA/DNA hybridisation experiments were carried out using RFLPs or ASO on restricted and non-restricted genomic DNA, but this has now been superseded, due to the widespread use of microsatellites, by PCR techniques that are more amenable to automation.

Obviously a detailed knowledge of the mutated site will be a necessity for the choice of the appropriate primers.

All the following methods enable the rapid screening of small areas of a coding region for the presence of unknown mutations. This is particularly useful if one wants to show that a particular 'candidate gene' for a genetic defect is indeed the 'culprit' for the occurrence of a particular disease.

1. Multiplex analysis
2. Single-stranded conformational analysis
3. Denaturing gradient gel electrophoresis

4. Chemical mismatch cleavage
5. Direct sequencing methods

The first four protocols have the disadvantage of saying nothing about the nature of the mutation; this can only be shown by DNA-sequencing of the area in which the mutation has been localised.

## **Multiplex Analysis**

In a first step several coding regions within a particular gene are amplified at the same time using a set of different primers flanking the exon/intron boundaries. This so called multiplex analysis enables the assessment of small deletion and/or insertion/amplification events as they occur at different sites along very large genes such as the DMD of CF gene; heterozygous individuals can be identified by a reduction of the band-intensities observed.

However point mutations cannot be detected via multiplex-PCR; instead one or several of the following procedures are used.

## **Conformational Analysis Single-stranded**

In a technique called single-stranded conformation analysis (SSCA) PCR amplified fragments of up to 200 bp are denatured and renatured very fast to avoid proper reannealing, then the sample is subject to electrophoresis on a non-denaturing polyacrylamide gel. Not only do the complementary strands migrate at different speeds but there is also a differential migration of one or both mutated strands. This differential migration can be used in the identification of a point mutation.

## **Denaturing (Temperature) Gradient Gel Electrophoresis (DGGE/TGGE)**

This technique involves the amplification of DNA by PCR followed by electrophoresis through a gradient of increasing concentration of a denaturing agent (such as urea or formamide) or temperature leading to a partial melting of the double stranded DNA around the  $T_m$  of

the mutated region. This results in an abrupt decrease in the mobility of the DNA-fragment. This assay requires the presence of both a wild-type allele and a mutant allele and is therefore very sensitive in identifying heterozygous carriers. Its sensitivity is good and its accuracy is claimed to be around 95% with a fragment length of up to 600 bp.

Direct heteroduplex analysis between wild type and mutated allele followed by electrophoresis on special matrices can also be used to identify point mutations.

## **Chemical Mismatch Cleavage (CMC)**

This technique uses heteroduplex molecules from PCR-products followed by chemical modification of the mismatched base using either  $\text{OsO}_4$  (for T) or  $\text{NH}_2\text{OH}$  (for C) and subsequent cleavage using piperidine and electrophoresis. This procedure allows the analysis of PCR-products in sizes up to 1.7 kbp.

### **The Methods of Direct DNA Sequencing**

There are basically three ways to rapidly sequence amplified DNA:

1. By asymmetric PCR using different molarities of the two primers
2. By biotinylating one of the two primers in order to remove one of the two strands by Streptavidin-coated magnetic beads
3. By cycle sequencing (also known as amplification sequencing), here fluorescence labeled dideoxynucleotides are added to the PCR/sequencing reaction and the products are separated on standard sequencing gels.

Note that both genomic and cDNA sequences can be screened for the presence of mutations, for the former exon-multiplexing is usually used in combination with SSCP or DDGE, for cDNA sequences CMC and direct sequencing tend to be the favourite techniques.

## **The Gene Therapy**

Genes are the blueprint for our bodies, governing factors such as

growth, development and functioning.

A genetic mutation means that a gene contains a fault or ‘spelling mistake’ that disrupts the gene message. A fault in a gene can occur spontaneously or can be inherited. Genetic faults can cause a wide range of disorders and be involved in susceptibility to some cancers. Gene therapy replaces faulty genes with healthy versions. Therapeutic gene therapy is still in its infancy, but has the potential to revolutionise treatment for all kinds of genetic diseases.

## **The Gene Therapy Process**

The basic steps of gene therapy include:

- The faulty gene that causes a specific disease must be identified
- The location of the affected cells must be pinpointed
- A healthy version of the gene must be available
- The healthy gene has to be delivered to the cell.

## **The Delivery Techniques**

The current problem is finding a way to successfully deliver the new gene. The *ex vivo* technique requires taking the affected cells from the person’s body and either ‘splicing’ or injecting the new genetic information into these cells. They are left to grow in the laboratory, and then replaced into the person. A promising technique is to put the healthy gene inside a deactivated virus, which has limited genetic information of its own. A virus that causes disease, such as the common cold, works by slipping into a cell, taking over its DNA and forcing it to produce more viruses. A deactivated virus can therefore enter the specific cell and deliver the healthy gene. Other techniques involve extracting stem cells from an individual’s bone marrow, putting the healthy gene into them and re-inserting the stem cell into their bone marrow. These stem cells can develop into a range of cell types.

## **Adenosine Deaminase (ADA) deficiency**

A person born with adenosine deaminase (ADA) deficiency lacks an important enzyme of their immune system. This means that infections are likely and can even be fatal. ADA deficiency was the first genetic disorder to undergo experimental gene therapy trials in 1990. It was chosen because it is caused by a single, relatively uncomplicated gene. The results were promising.

## **The Immune System**

Current research is focusing on the immune system, which is a collection of special cells and chemicals that fight infection. If the immune system isn't functioning in the right way, illness can result. One theory on cancer suggests that the immune system is failing to stop the overgrowth of cells that form a tumour. If the immune system could be 'bolstered' with gene therapy, perhaps the body would be able to prevent the spread of cancer by itself. One day, gene therapy may also be used as a form of immunisation against particular infections, such as HIV/AIDS and malaria.

Another condition where gene therapy is promising is called X-linked severe combined immune deficiency (X-SCID). Children affected by X-SCID have a faulty gene that means they have no working immune system, so their bodies cannot fight infections. Only boys are affected due to the pattern of inheritance of the faulty gene. Until recently, unless they could be given a matched bone marrow transplant, boys with X-SCID faced a lifetime living in a sterile bubble. With gene therapy, bone marrow from the boys is first harvested. The white blood stem cells from the bone marrow are then infected with a virus carrying a working gene, before returning the cells to the boys' bodies. This treatment has worked to successfully restore the immune system in several children. However there have been a number of concerns related to the treatment's safety.

## **Body Cells Versus Reproductive Cells**

A replaced, healthy gene would cure the individual, but not prevent

their children from inheriting the original faulty gene, which is carried on the sperm and ova (germ cells). While the body's other cells (somatic cells) would have the updated healthy gene, the germ cells would still contain the defective one. To make sure that future generations of the person were healthy, their germ cells would need to undergo gene therapy too. However, a complicated range of ethical issues, as well as technical problems, means that gene therapy of germ cells is only a remote possibility.

## **The Risks of Gene Therapy**

Some of these risks may include:

- The immune system may respond differently than expected to the new gene and cause inflammation.
- The healthy gene might be slotted into the wrong spot.
- The healthy gene might produce too much missing enzyme or protein, causing other health problems.
- Other genes may be accidentally delivered to the cell.
- The deactivated virus might target other cells as well as the intended cells.
- The deactivated virus may be contagious.

While these risks have not been observed in gene therapy tested on animals there are still concerns over the safety of the techniques.

## **Genetic Engineering and the Ethics**

The gene offers a range of complex ethical and moral dilemmas. Some people believe that gene therapy is the same thing as genetic engineering. Currently, genetic engineering is concerned with altering food crops, while gene therapy aims to eliminate disease at its source, not produce a 'better' class of human being. However, the concern is that manipulating factors such as intelligence might be tried, once gene therapy becomes commonplace. 'Ordinary' characteristics, such



as shortness or average IQ, might then be considered as 'subnormal'. Another concern is that gene therapy might only be available to the rich. The challenge for nations experimenting with gene therapy is to come up with workable, fair and ethical guidelines for its use. However gene therapy is an experimental form of treatment that works by replacing a faulty disease-causing gene with a healthy version. The aim is to eliminate genetic diseases at their source. The challenge for nations experimenting with gene therapy is to come up with workable, fair and ethical guidelines for its use.

## **What is Gene Therapy?**

Genes, which are carried on chromosomes, are the basic physical and functional units of heredity. Genes are specific sequences of bases that encode instructions on how to make proteins. Although genes get a lot of attention, it's the proteins that perform most life functions and even make up the majority of cellular structures. When genes are altered so that the encoded proteins are unable to carry out their normal functions, genetic disorders can result.

Gene therapy is a technique for correcting defective genes responsible for disease development.

## **How to Correct Faulty Genes?**

- A normal gene may be inserted into a non-specific location within the genome to replace a non-functional gene. This approach is most common.
- An abnormal gene could be swapped for a normal gene through homologous recombination.
- The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.
- The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.

## The Workings of Gene Therapy

In most gene therapy studies, a 'normal' gene is inserted into the genome to replace the abnormal, disease-causing gene. A carrier molecule called a vector must be used to deliver the therapeutic gene to the patient's target cells. Currently, the most common vector is a virus that has been genetically altered to carry normal human DNA. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have tried to take advantage of this capability and manipulate the virus genome to remove disease-causing genes and insert therapeutic genes. Target cells such as the patient's liver or lung cells are infected with the viral vector. The vector then unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state. Some of the different types of viruses used as gene therapy vectors:

- Retroviruses - A class of viruses that can create double-stranded DNA copies of their RNA genomes. These copies of its genome can be integrated into the chromosomes of host cells. Human immunodeficiency virus (HIV) is a retrovirus.
- Adenoviruses - A class of viruses with double-stranded DNA genomes that cause respiratory, intestinal and eye infections in humans. The virus that causes the common cold is an adenovirus.
- Aden-associated viruses - A class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.
- Herpes simplex viruses - A class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.

Besides virus-mediated gene-delivery systems, there are several nonverbal options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can be used only with certain tissues and requires large amounts of DNA.

Another nonviral approach involves the creation of an artificial lipid sphere with an aqueous core. This liposome, which carries the therapeutic DNA, is capable of passing the DNA through the target cell's membrane.

Therapeutic DNA also can get inside target cells by chemically linking the DNA to a molecule that will bind to special cell receptors. Once bound to these receptors, the therapeutic DNA constructs are engulfed by the cell membrane and passed into the interior of the target cell. This delivery system tends to be less effective than other options.

Researchers also are experimenting with introducing a 47th (artificial human) chromosome into target cells. This chromosome would exist autonomously alongside the standard 46—not affecting their workings or causing any mutations. It would be a large vector capable of carrying substantial amounts of genetic code and scientists anticipate that, because of its construction and autonomy, the body's immune systems would not attack it. A problem with this potential method is the difficulty in delivering such a large molecule to the nucleus of a target cell.

## **Current status of Gene Therapy Research**

The Food and Drug Administration (FDA) has not yet approved any human gene therapy product for sale. Current gene therapy is experimental and has not proven very successful in clinical trials. Little progress has been made since the first gene therapy clinical trial began in 1990. In 1999, gene therapy suffered a major setback with the death of 18-year-old Jesse Gelsinger. Jesse was participating in a gene therapy trial for ornithine transcarboxylase deficiency (OTCD). He died from multiple organ failures 4 days after starting the treatment. His death is believed to have been triggered by a severe immune response to the adenovirus carrier.

Another major blow came in January 2003, when the FDA placed a temporary halt on all gene therapy trials using retroviral vectors in blood stem cells. FDA took this action after it learned that a second child treated in a French gene therapy trial had developed a leukemia-

like condition. Both this child and another who had developed a similar condition in August 2002 had been successfully treated by gene therapy for X-linked severe combined immunodeficiency disease (X-SCID), also known as ‘bubble baby syndrome.’

FDA’s Biological Response Modifiers Advisory Committee (BRMAC) met at the end of February 2003 to discuss possible measures that could allow a number of retroviral gene therapy trials for treatment of life-threatening diseases to proceed with appropriate safeguards. FDA has yet to make a decision based on the discussions and advice of the BRMAC meeting.

## **Why Gene Therapy is not an Effective Treatment for Genetic Disease**

- Short-lived nature of gene therapy - Before gene therapy can become a permanent cure for any condition, the therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving any long-term benefits. Patients will have to undergo multiple rounds of gene therapy.
- Immune response - Anytime a foreign object is introduced into human tissues, the immune system is designed to attack the invader. The risk of stimulating the immune system in a way that reduces gene therapy effectiveness is always a potential risk. Furthermore, the immune system’s enhanced response to invaders it has seen before makes it difficult for gene therapy to be repeated in patients.
- Problems with viral vectors - Viruses, while the carrier of choice in most gene therapy studies, present a variety of potential problems to the patient —toxicity, immune and inflammatory responses and gene control and targeting issues. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease.

- **Multigene disorders** - Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis and diabetes, are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy.

## Some Recent Developments in Gene Therapy

University of California, Los Angeles, research team gets genes into the brain using liposomes coated in a polymer call polyethylene glycol (PEG). The transfer of genes into the brain is a significant achievement because viral vectors are too big to get across the 'blood-brain barrier.' This method has potential for treating Parkinson's disease.

- RNA interference or gene silencing may be a new way to treat Huntington's. Short pieces of double-stranded RNA (short, interfering RNAs or siRNAs) are used by cells to degrade RNA of a particular sequence. If a siRNA is designed to match the RNA copied from a faulty gene, then the abnormal protein product of that gene will not be produced.
- New gene therapy approach repairs errors in messenger RNA derived from defective genes. Technique has potential to treat the blood disorder thalassaemia, cystic fibrosis and some cancers.
- Gene therapy for treating children with X-SCID (sever combined immunodeficiency) or the 'bubble boy' disease is stopped in France when the treatment causes leukemia in one of the patients.
- Researchers at Case Western Reserve University and Copernicus Therapeutics are able to create tiny liposomes 25 nanometers across that can carry therapeutic DNA through pores in the nuclear membrane.
- Sickle cell is successfully treated in mice.

## **Carcinogenesis**

Carcinogenesis is agents that can cause cancer. In industry there are many potential exposures to carcinogenesis. Generally workplace exposures are considered to be at higher levels than for public exposures.

### **The Chemical Carcinogenesis**

This is a multi-stage process that begins with exposure, usually to complex mixtures of chemicals that are found in the human environment. Once internalised, carcinogens frequently are subjected to competing metabolic pathways of activation and detoxication, although some reactive environmental chemicals can act directly. Variations among individuals in the metabolism of carcinogens, together with differences in DNA-repair capacity and response to tumour promoters, govern the relative risk of an individual. The initial genetic change that occurs as the result of chemicalDNA interaction is termed tumour initiation. Thus, initiated cells are irreversibly altered and are at greater risk of malignant conversion than are normal cells. The epigenetic effects of tumour promoters facilitate the clonal expansion of the initiated cell. This selective, clonal growth advantage results in the formation of a focus of preneoplastic cells. These cells are more vulnerable to progress toward tumourigenesis because they present a larger, more rapidly proliferating target population for the further action of chemical carcinogens, oncogenic viruses and other cofactors. Additional genetic changes occur and consequently, the

accumulation of mutations, which may activate proto-oncogenes and inactivate tumour-suppressor genes, leads to malignant conversion, tumour progression and metastasis. The underlying genetic mechanisms that regulate chemical carcinogenesis are becoming increasingly well understood and the insights generated have assisted in the development of methodologies designed to assess human cancer risk and susceptibility factors. The results of these latter studies are further intended to mould strategies for cancer prevention.

## Multi-stage Carcinogenesis

During the last 50 years, the sequence of events comprising chemical carcinogenesis has been systematically dissected and the paradigm increasingly refined. It is now recognised that carcinogenesis requires the malignant conversion of hyperplastic cells from a benign to malignant state and that invasion and metastasis are manifestations of further genetic and epigenetic changes. Study of this process in humans is necessarily indirect. Measures of age-dependent cancer incidence have shown, however, that the rate of tumour development is proportional to the sixth power of time, suggesting that at least four to six independent steps are necessary. Partial scheduling of specific genetic events in this process for some cancers has been possible. Sequential genetic changes occurring during the development of cancer of the head and neck or colon cancer are examples.

Carcinogenesis can be divided conceptually into four steps:

1. Tumour initiation
2. Tumour promotion
3. Malignant conversion
4. Tumour progression

The distinction between initiation and promotion was recognised through studies involving both viruses and chemical carcinogens. This distinction was formally defined in a murine skin carcinogenesis model where mice were treated topically with a single dose of a polycyclic aromatic hydrocarbon (i.e., initiator), followed by repeated topical

doses of croton oil (i.e., promoter). This mechanism also has been shown to operate in a range of other rodent tissues, including the bladder, colon, esophagus, liver, lung, mammary gland, stomach and trachea

### **1. Tumour Initiation**

Tumour initiation results from irreversible genetic damage. For mutations to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. A chemical carcinogen causes a genetic error by modification of the molecular structure of DNA that can lead to a mutation during DNA synthesis. Most often, this is brought about by formation of an adduct between the chemical carcinogen or one of its functional groups and a nucleotide in DNA. (The process by which this occurs for the major classes of chemical carcinogens is discussed in detail under Carcinogen Metabolism). In general, a positive correlation is found between the amount of carcinogenDNA adducts that can be detected in animal models and the resulting number of tumours that develop. Thus, tumours rarely develop in tissues that do not form carcinogenDNA adducts. CarcinogenDNA adduct formation is central to theories of chemical carcinogenesis and it can be considered to be a necessary, but not a sufficient, prerequisite for tumour initiation. DNA adducts formation that results in either the activation of a proto-oncogene or the inactivation of a tumour suppressor gene can be considered to be a tumour-initiating event.

### **2. Tumour Promotion**

Tumour promotion comprises the selective clonal expansion of initiated cells. Because the accumulation rate of mutation is proportional to the rate of cell division or at least the rate at which stem cells are replaced, it follows that clonal expansion of initiated cells produces a larger population of cells that are at risk of further genetic changes and malignant conversion. Tumour promoters generally are nonmutagenic, not carcinogenic alone and often (but not always) able to mediate their biologic effects without metabolic activation. These agents are characterised by their ability to reduce the latency period for tumour formation after exposure of a tissue to a tumour



initiator or to increase the number of tumours formed in that tissue. In addition, they induce tumour formation in conjunction with a dose of an initiator that is too low to be carcinogenic alone. Chemicals or agents capable of both tumour initiation and promotion are known as complete carcinogens, examples are benzo [a]pyrene and 4-aminobiphenyl.

Croton oil (isolated from Croton Tiglium seeds) has been used widely as a tumour promoter in murine skin carcinogenesis and the mechanism of action for its most potent constituent, 12-tetradecanoylphorbol-13-acetate, via activation of protein kinase C is arguably the best understood among tumour promoters. Protein kinase C is a calcium-phospholipid-dependent enzyme family that, when activated, causes phosphorylation of critical substrates and stimulates a cascade of epigenetic changes that can lead to cell growth. Among the changes observed in cells treated with 12-O-tetradecanoylphorbol-13-acetate is altered ion flux across the cell membrane, altered hormone binding and inhibition of cell-cell communication. With increasing recognition of redundancy in the signal transduction cascade, however, it is possible to appreciate that the effects of 12-O-tetradecanoylphorbol-13-acetate are even more diverse. Prostaglandin synthesis, which also is associated with tumour promotion, occurs because of stimulation of the arachidonic acid cascade that is mediated by protein kinase C. The cellular response to protein kinase C activation can result in the modification of differentiation or cell proliferation and is cell-type dependent. The cell-type dependent differential response may be explained by the fact that protein kinase C is a multi-gene family, the members of which are differentially expressed among animal species and tissue types.

Identification of new tumour promoters in animal models has accelerated with the increasingly sophisticated development of model systems designed to assay for tumour promotion. Furthermore, ligand-binding properties also can be determined in recombinant protein kinase C isozymes that are expressed in cell cultures. Chemicals, complex mixtures of chemicals or other agents that have been shown to have tumour-promoting properties include dioxin, benzoyl

peroxide, macrocyclic lactones, bromomethylbenz anthracene, anthralin, phenol, saccharin, tryptophan, dichlorodiphenyltrichloroethane (DDT), phenobarbital, cigarette-smoke condensate, polychlorinated biphenyls (PCBs), teleocidins, cyclamates, estrogens and other hormones, bile acids, ultraviolet light, wounding, abrasion and other chronic irritations (i.e., saline lavage). It also has been noted that protein kinase C is activated and cellular diacylglycerol elevated in laboratory animals maintained on high-fat diets.

### 3. Malignant Conversion

Malignant conversion is the transformation of a preneoplastic cell into one that expresses the malignant phenotype. This process requires further genetic changes. The total dose of a tumour promoter is less important than frequently repeated administrations and if the administration of a tumour promoter is discontinued before malignant conversion has occurred, premalignant or benign lesions may regress. The contribution of tumour promotion to the process of carcinogenesis is the expansion of a population of initiated cells, which will then be at risk for malignant conversion. Conversion of a fraction of these cells to malignancy will be accelerated in proportion to the rate of cell division and the quantity of dividing cells in the benign tumour or preneoplastic lesion. In part, these further genetic changes may result from infidelity of DNA synthesis. The relatively low probability of malignant conversion can be increased substantially by the exposure of preneoplastic cells to DNA-damaging agents and this process may be mediated through the activation of proto-oncogenes and inactivation of tumour-suppressor genes.

### 4. Tumour Progression

Tumour progression comprises the expression of the malignant phenotype and the tendency of already malignant cells to acquire more aggressive characteristics with time. Metastasis also may involve the ability of tumour cells to secrete proteases that allow invasion beyond the immediate location of the primary tumour. A prominent characteristic of the malignant phenotype is the propensity for genomic instability and uncontrolled growth. During this process further genetic

changes can occur, again including the activation of proto-oncogenes and the functional loss of tumour-suppressor genes. Proto-oncogenes frequently are activated by two major mechanisms: in the case of the ras gene family, point mutations are found in highly specific regions of the gene (i.e., the 12th, 13th, 59th or 61st codons) and members of the myc, raf, her-2 and jun multi-gene families can be over expressed, sometimes involving amplification of chromosome segments containing these genes. Some genes are over expressed if they are translocated and become juxtaposed to a powerful promoter (e.g., the relationship of bcl-2 and immunoglobulin gene promoter regions in B-cell malignancies). Loss of function of tumour-suppressor genes usually occurs in a bimodal fashion and most frequently involves point mutations in one allele and loss of the second allele by deletion, recombination event or chromosomal nondisjunction. These phenomena confer to the cells a growth advantage as well as the capacity for regional invasion and, ultimately, distant metastatic spread. Despite evidence for an apparent scheduling of certain mutational events, it is the accumulation of these mutations and not the order or the stage of tumourigenesis in which they occur, that appears to be an important determining factor.

Activation of proto-oncogenes and inactivation of tumour suppressor genes are mutational events that occur as the result of covalent damage to DNA caused by chemical exposures. The accumulation of mutations and not necessarily the order in which they occur constitutes multistage carcinogenesis.

## **Interindividual Variation and Gene Environment Interaction**

Gene environment interaction is the cornerstone of human chemical carcinogenesis. Potential interindividual susceptibility to chemical carcinogenesis may well be defined by genetic variations in the host elements of this compound system. Functional polymorphisms, among humans, of proteins that have or may have, a role in chemical carcinogenesis include enzymes that metabolise (i.e., activate and detoxify) xenobiotic substances, enzymes that repair DNA damage,

cell surface receptors that activate the phosphorylation cascade and cell cycle control genes (i.e., oncogenes and tumour suppressor genes that are elements of the signal transduction cascade).

When chemicals or xenobiotics encounter biologic systems, they become altered by metabolic processes. This is an initial facet of the gene-environment interaction. Interindividual variation in carcinogen metabolism and macromolecular adduct formation arises from such processes and was recognised 20 to 25 years ago. The cytochrome P-450 (CYP) multi-gene family is largely responsible for the metabolic activation and detoxication of many different chemical carcinogens in the human environment. Cytochrome P-450s are phase I enzymes that act by adding an atom of oxygen onto the substrate, they also are inducible by polycyclic aromatic hydrocarbons and chlorinated hydrocarbons. Phase II enzymes act on oxidised substrates and also contribute to xenobiotic metabolism. Some phase II enzymes are methyltransferases, acetyltransferases, glutathione transferases, uridine 5'-diphosphoglucuronosyl transferases, sulfotransferases, nicotinamide-adenine dinucleotide (NAD)- and nicotinamide-adenine dinucleotide phosphate (NADP)-dependent alcohol, aldehyde and steroid dehydrogenases, quinone reductases, NADPH diaphorase, azo reductases, aldoketoreductases, transaminases, esterases and hydrolases. The pathways of activation and detoxication are often competitive, providing yet further potential for individual differences in propensity for carcinogen metabolism. This scenario is further complicated by a process of enzyme induction or inhibition, where genes responsible for carcinogen metabolism can be upregulated or repressed by certain chemical exposures.

When the chemical alters the gene a second facet of gene-environment interaction occurs. Once a procarcinogen has been metabolically activated to an ultimate carcinogenic form, it can bind covalently to cellular macromolecules, including DNA. This damage to DNA can be repaired by several mechanisms. Differences in the rates and fidelity of DNA repair potentially influence the extent of carcinogen adduct formation (i.e., biologically effective dose) and, consequently, the total amount of genetic damage that accumulates. The consequence of

polymorphisms in genes controlling the cell cycle (serine/threonine kinases, transcription factors, cyclins, cyclin-dependent kinase inhibitors and cell surface receptors) is much less clear. However, molecular epidemiologic evidence suggests that certain common variants of these types of gene have a role in susceptibility to chemical carcinogenesis. Evaluation of polymorphisms as potential biomarkers of susceptibility in the human population are discussed under Molecular Epidemiology.

## **Metabolism of the Carcinogen**

The first chemically identified carcinogens were the polycyclic aromatic hydrocarbons. They are composed of variable numbers of fused benzene rings that form from incomplete combustion of fossil fuels and vegetable matter and they are common environmental contaminants. The polycyclic aromatic hydrocarbons are chemically inert and require metabolism to exert their biologic effects. This is a multi-step process, involving initial epoxidation (cytochrome P-450, CYP1A1 an inducible isoform), hydration of the epoxide (epoxide hydrolase) and subsequent epoxidation across the remaining olefinic bond (principally CYP3A4). The result is the formation of the ultimate carcinogenic metabolite, a diol-epoxide. Variations among humans in the levels of the enzymes involved has been documented. The biology of CYP1A1 metabolism has been elucidated, providing a molecular basis for inducibility and interindividual variation. Metabolic activation of benzo [a] pyrene leads to the formation of the bay-region benzo [a] pyrene-7, 8-diol 9,10-oxide. This vicinal diol-epoxide is asymmetric and eight stereoisomers are possible. The reactivity of each isomer is variable and the isomers are formed in varying proportions by metabolism. Biologic response to the different enantiomers in mammalian systems suggests that the anti forms are the most active mutagens and carcinogens and the syn forms are the least active. The arene ring of benzo [a] pyrene-7, 8-diol 9,10-oxide opens spontaneously at the 10-position, giving a highly reactive carbonium ion that can form a covalent addition product (i.e., adduct) with cellular macromolecules, including DNA. These adduct cause the DNA

to be damaged, either by their persistence and consequent interference with replication or by aberrant DNA repair. The same basic tenet holds for carcinogen DNA adducts of other chemical classes that may be activated by different metabolic pathways.

Another class of chemical carcinogenesis are the Aromatic amines. This and 4-aminobiphenyl are thought to be responsible for bladder cancer among workers in the rubber industry. This and many related compounds are components of cigarette smoke, diesel exhaust and the pyrolysis of certain foods. In addition, nitrated polycyclic aromatic hydrocarbons are also environmental contaminants resulting from the incomplete combustion of vegetable matter and diesel fuel and they are related to aromatic amines by nitroreduction. The metabolic activation of aromatic amines is complex. They can be converted to an aromatic amide that is catalysed by an acetyl coenzyme a dependent acetylation. The acetylation phenotype varies among the population. Persons with the rapid acetylator phenotype are at higher risk of colon cancer, whereas those who are slow acetylators are at risk of bladder cancer. This latter association may result from the fact that activation of aromatic amines by N-oxidation is a competing pathway for aromatic amine metabolism. Also the N-hydroxylation products, when protonated (by the acid conditions in the urinary bladder), form reactive electrophiles that bind covalently with DNA or proteins to produce macromolecular damage. An initial activation step for both aromatic amines and amides is N-oxidation by CYP1A2.

This cytochrome P-450 is inducible by phenobarbital and because it is also responsible for the 3-demethylation of 1,3,7-trimethylxanthine (i.e., caffeine), the distribution of metabolic phenotypes in the population, as well as the disposition of an individual with respect to CYP1A2 metabolism, is relatively easy to determine. The reaction of N-hydroxy-arylamines with DNA appear to be acid catalysed, but they can be further activated by either an acetyl coenzyme A dependent O-acetylase or a 3'-phosphoadenosine-5'phosphosulfatedependent O-sulfotransferase. The N-arylhydroxamic acids, which arise from the acetylation of N-hydroxy-arylamines or N-hydroxylation of aromatic amides, are not electrophilic; therefore, they require further activation.

The predominant pathway for this occurs through acetyltransferase-catalysed rearrangement to a reactive N-acetoxy-arylamine. Sulfotransferase catalysis results in the formation of N-sulfonyloxy arylamides. This complex pathway results in two major adduct types: amides (i.e., acetylated) and amines (i.e., nonacetylated). The heterocyclic amines are formed during the preparation of cooked food, primarily from the pyrolysis ( $> 150^{\circ}\text{C}$ ) of amino acids, creatinine and glucose. They have been recognised as food mutagens and they have been shown to form DNA adducts and cause liver tumours in primates. Compared with other carcinogens, their metabolism is less well understood, but N-hydroxylation is considered to be a necessary step. Because they are similar in structure to the aromatic amines, it is not surprising that they can be activated by CYP1A2. The N-hydroxy metabolites of 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-1), 2-amino-6-methyldipyrido [1,2-a: 3,9-d] imidazole (Glu-P-1) and 2-amino-3-methyl-imidazo-[4,5-f]quinoline (IQ) can react directly with DNA. Unlike, however, the aromatic amines, this reaction is not facilitated by acid pH. Enzymic O-esterification of N-hydroxy metabolites is important in the activation of these food mutagens and the N-hydroxy metabolites also are good substrates for transacetylases. This suggests a possible role for these chemicals in the etiology of colorectal cancer in combination with the rapid acetylator phenotype. Aflatoxins (aflatoxin B1, B2, G1 and G2) are metabolites of *Aspergillus flavus*. These fungal mutagens contaminate improperly stored cereals, grains and nuts. A positive correlation exists between dietary aflatoxin exposure and incidence of liver cancer in the developing countries, where grain spoilage is high. Aflatoxins are activated by several cytochrome P-450s, including CYP2A3, CYP2A6 and CYP3A4. Aflatoxin B1 and G1 have an olefinic double bond at the 8,9-position and they are more mutagenic and carcinogenic than aflatoxin B2 and G2, which are saturated and have an ethylenic bond at this position. This implies that the olefinic 8,9-bond is the site of activation. Further support for this mechanism comes from studies of DNA adducts and the prevalence of p53 mutations in liver cancer. In people with liver cancer from parts of China and Africa where food spoilage caused by molds is high, G: C to T: A transversions in codon 249 are frequent.

This phenomenon is consistent with metabolic activation of aflatoxin B<sub>1</sub>. Carcinogenic N-nitrosamines are ubiquitous environmental contaminants and can be found in food, alcoholic beverages, cosmetics, cutting oils, hydraulic fluid, rubber and tobacco. Tobacco-specific N-nitrosamines such as 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone are carcinogenic in a wide range of animal species and they may account for the carcinogenic nature of snuff and chewing tobacco. Endogenous nitrosation also can occur because of the reaction of an amine with nitrate alone or nitrite in the presence of acid. Thus, nitrite (used in curing meats) and L-cysteine in the presence of acetaldehyde (a metabolite of alcohol) form N-nitrosothiazolidine-4-carboxylic acid. The N-nitrosamines are activated primarily by CYP2E1, which is inducible by alcohol. N-nitrosodimethylamine undergoes a-hydroxylation to form an unstable a-hydroxynitrosamine. The breakdown products are formaldehyde and methyl diazohydroxide. The alkyl groups of compounds, such as methyl diazohydroxide, are good leaving groups and, thus, are powerful methylating agents that can add a small functional group (small alkyl adduct as opposed to the bulky aryl adducts formed by the carcinogens discussed earlier) at more than 10 different sites in DNA. The tobacco-specific nitrosamines are not symmetric and also can form bulky adducts, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone metabolism gives rise to either a positively charged pyridyl-oxobutyl ion or a positively charged methyl ion, both of which are able to alkylate DNA.

Metabolic activation of benzo [a] pyrene. (1) Cytochrome P-450 (CYP1A1) catalyses initial epoxidation across the 1 - 2, 2 - 3, 4 - 5, 7 - 8 (shown), 9 - 10 and 11 - 12 positions. (2) With the exception of the 1 - 2 and 2 - 3 oxides which convert to phenols, epoxide hydrolase catalyses the formation of dihydrodiols. (3) Benzo [a] pyrene-7, 8-dihydrodiol is further metabolised at the olefinic double bond by cytochrome P450 (CYP1A1 and CYP3A4) to form a vicinal diol-epoxide (r7, t8-dihydroxy-c9, 10 epoxy-7, and 8,9,10-tetrahydroxybenzo [a] pyrene). (4) The highly unstable arene ring opens spontaneously to form a carbocation. (5) This electrophilic species forms a covalent bond between the 10 positions of the hydrocarbon and the exocyclic amino group of deoxyguanosine.



## DNA Damage and Repair

There are several ways in which the chemical structure of DNA can be altered by a carcinogen, including the formation of bulky aromatic-type adducts, alkylation (generally small adducts), oxidation, dimerisation and deamination. Chemical carcinogens also can cause epigenetic changes, such as alteration in DNA methylation status that can lead to silencing of specific gene expression. Carcinogen-DNA adducts vary in their promutagenic potential, the binding of benzo [a] pyrene-7,8-diol 9,10-epoxide to the exocyclic (N2) amino group of deoxyguanosine forms an aromatic adduct that resides within the minor groove of the double helix and it is typical of polycyclic aromatic hydrocarbons. Although this adduct appears to be the most common form, by far, of DNA damage induced by benzo [a] pyrene in mammalian systems, others are possible, including covalent binding of metabolites to deoxyadenosine. Aromatic amine adducts are more complex, not only because they have both acetylated and nonacetylated metabolic intermediates but also because they form covalent bonds at the C8-, N2- and sometimes O6-positions of deoxyguanosine as well as deoxyadenosine. The major adducts, however, are C8-deoxyguanosine adducts, which reside predominantly in the major groove of the DNA double helix.

Although the evidence for the activation of aflatoxins B1 and G1 through hydroxylation of the olefinic 8,9-position is circumstantial, the structures of the adducts are known. They are formed at the N7-position of deoxyguanosine. They are relatively unstable and have a half-life of approximately 50 hours at neutral pH, with resulting depurination. The aflatoxin B1-N7-deoxyguanosine adduct also can undergo ring opening, to yield two pyrimidine adducts, alternatively, aflatoxin B1-8,9-dihydrodiol could result. This latter possibility could restore the molecular structure of DNA if hydrolysis of the original adduct occurs, but a potentially promutagenic lesion would result if formation of the 8,9-dihydrodiol results from degradation of ring-open adduct forms.

Alkylation of DNA can occur at many sites, either following the

metabolic activation of certain N-nitrosamines or directly by the action of the N-alkylureas (N-methyl-N-nitrosourea) or the N-nitrosoguanidines. The protonated alkyl-functional groups that become available to form lesions in DNA generally attack the following nucleophilic centres: adenine (N1, N3 and N7), cytosine (N3), guanine (N2, O6 and N7) and thymine (O2, N3 and O4). Some of these lesions are known to be repaired (O6-methyldeoxyguanosine), while others are not (N7-methyldeoxyguanosine). Furthermore, O6-methyldeoxyguanosine is a promutagenic lesion, whereas N7-methyldeoxyguanosine is not. Oxy-radical damage can result in the modification of DNA to form thymine glycol or 8-hydroxydeoxyguanosine adducts. Three major pathways have been identified. Exposure to organic peroxides (catechol, hydroquinone and 4-nitroquinoline-N-oxide) leads to this type of oxyradical damage; however, oxyradicals and hydrogen peroxide can be generated in lipid peroxidation and the catalytic cycling of some enzymes. Cells also can be stimulated to produce peroxisomes by treatment with certain drugs and plasticisers. Exposure to tumour promoters can indirectly increase oxyradical formation and perhaps the best known relationship is that between the phorbol esters and inflammatory cells. In this system, mediated through protein kinase C and the subsequent activation of a membrane-localised pyridine nucleotide-dependent oxidase, oxyradical formation is highly correlated with the relative potencies of the different phorbol esters. Correspondingly, promoters that do not stimulate the protein kinase C signal transduction cascade do not affect oxyradical production. Another potentially mutagenic cause of DNA damage is the deamination of methylated cytosine residues in DNA. 5-methylcytosine comprises approximately 3% of deoxynucleotides. In this case, deamination at a CpG dinucleotide gives rise to a TpG mismatch. Repair of this lesion most often restores the CpG, however, repair may also cause a mutation (TpA). Deamination of cytosine also can generate a C-to-T transition, if uracil glycosylation and G-T mismatch repair are inefficient. Oxyradicals can enhance the rate of deamination, so the activity of inducible nitric oxide synthase and production of high concentrations of nitric oxide could contribute to DNA damage by this mechanism.

DNA repair enzymes act at sites of DNA damage caused by chemical carcinogens and six major mechanisms are known: direct DNA repair, nucleotide excision repair, base excision repair, double-strand break repair, mismatch repair and postreplication repair. These have been characterised in lower organisms, such as yeast and bacteria. However, among recent advances are the cloning of more than 70 human genes involved in five of these DNA repair pathways. These genes are responsible for the fidelity of DNA repair and defects in these genes provides a biologic mechanism for the genetic defect leading to increased mutations or the mutator phenotype.

Direct DNA repair is affected by DNA-alkyltransferases. These enzymes catalyse translocation of the alkyl moiety from an alkylated base (e.g., O6-methyldeoxyguanosine) to a cysteine residue at their active site, in the absence of DNA strand scission. Thus, one molecule of the enzyme is capable of repairing one alkyl lesion in DNA. The human O6-methyldeoxyguanosine DNA-methyltransferase has been mapped to chromosome 10q24.33 - qter.

In nucleotide excision repair of DNA, lesion recognition, preincision, incision, gap-filling and ligation are required. Therefore, it is not surprising that the so-called excinuclease complex comprises 16 or more different proteins. Large distortions caused by bulky DNA adducts (e.g., BPDE-dG and AAF-dC) are recognised by specific proteins that recognise DNA damage (e.g., XPA). Removal of the DNA damage is achieved by the action of the endonucleases (e.g., XPE, XPG and FEN). Then using the intact strand as a template, a patch is constructed by 5' to 3' polymerisation and ligation of the free ends then occurs. This type of transcription repair is strand specific that is, the transcribed strand in a gene is preferentially repaired by comparison to the non transcribed or DNA coding strand. Nucleotide excision repair is a vital mechanism in humans and lack of this function results in xeroderma pigmentosum (XP).

Base excision repair also removes a segment of DNA containing an adduct, however, small adducts (e.g., 3-methyladenine) are generally the target so that there is overlap with direct repair. Removal of the adducted base is brought about by a glycosylase (e.g., hOgg1, UDG)

and repair of the damaged strand is accomplished by the combined action of an apurinic endonuclease (e.g., HAP1) that degrades a few bases on the damaged strand and a polymerase that synthesises a 'patch' in the 5' to 3' direction, using the undamaged strand as a template (e.g., pol). The patch is then ligated by one of a number of ligases (DNA ligases: I, II, III, III IV).

DNA mismatches occasionally occur because of excision repair processes and involve incorporation of unmodified or conventional, but noncomplementary, Watson-Crick bases opposite each other in the DNA helix. Transition mispairs (G-T or A-C) are repaired by the mismatch repair process more efficiently than transversion mispairs (G-G, A-A, G-A, C-C, C-T and T-T), probably because of differential recognition of the mispairings. Repair efficiency of mispairings also depends on their oligonucleotide environment for the same reason. Thus, mispairings in the G-Crich regions are repaired more efficiently than those in the A-Trich regions. The mechanism for the correction of mispairings is similar to that for nucleotide excision repair and resynthesis described earlier, but it generally involves the excision of large pieces of the DNA containing mispairings. Because the mismatch recognition protein is required to bind simultaneously the mismatch and an unmethylated adenine in a G-A-T-C recognition sequence, it removes the whole intervening DNA sequence. The parental template strand is then used by the polymerase to fill the gap. Relatively common mutations in hPMS1, hPMS2, hMLH1, hMSH2, hMSH3 have been shown to predispose to human hereditary nonpoliposis colon cancer and glioma. Double-strand DNA breaks can occur from exposure to ionising radiation and oxidation. Consequences of double-strand DNA breaks are inhibition of replication and transcription and loss of heterozygosity. Double-strand DNA break repair occurs through homologous recombination, joining of the free ends is mediated by a DNA-protein kinase in a process that also protects the ends from nucleolytic attack. The free ends of the DNA then undergo ligation by DNA ligase IV. Genes known to code for DNA-repair enzymes that participate in this process include: xrc4, xrc5, xrc6, xrc7, hrad51b, hrad52, RPA and ATM.

The mechanism of post-replication is damage tolerant and it occurs in response to replication of DNA on a damaged template. The DNA polymerase stops at the replication fork when DNA damage is detected on the parental strand. Alternatively, the polymerase proceeds past the lesion, leaving a gap in the newly synthesised strand. The gap is filled in one of two ways: either by recombination of the homologous parent strand with the daughter strand in a process that is mediated by the RecA protein or, when a single nucleotide gap remains, mammalian DNA polymerases insert an adenine residue. Consequently, this mechanism may lead to recombinational events as well as base mispairing.

The rate, but not the fidelity, of DNA repair can be measured by adduct removal or unscheduled DNA synthesis. Substantial variations among individuals have been found in these rates. Markedly reduced rates of excision repair are found in individuals with xeroderma pigmentosum and these individuals are at known risk of ultraviolet lightinduced skin cancer. Among the general population, however, an approximately five-fold variation in the rates of excision repair have been found in lymphocytes treated with carcinogens in vitro. An association also has been found between the reduced capacity of mononuclear leukocytes in vitro to repair aromatic amine adducts in individuals who have first-degree relatives with cancer. Up to 40-fold variations among humans in the activity of O6-alkylguanine-DNA alkyltransferase have been reported as well. DNA repair rates are inhibited by aldehydes, alkylating agents and some chemotherapeutic drugs. Decreased DNA repair capacity also has been noted in the fibroblasts of patients with lung cancer compared with those patients with melanoma or noncancer controls. For benzo [a] pyrene-7, 8-diol 9,10-epoxideDNA adducts, a unimodal distribution of repair rates is observed in lymphocytes, but interindividual variation has been found to be substantial.

Computer modeled image of anti-benzo [a] pyrene-diol epoxide deoxyguanosine adduct formed in the minor groove of a 12-base pair sequence (5'-ATCggCgCggTA-3'). The structure is a half-bond colour stick model.

## **The Tumour Promoters and Biologic Responses**

Different biological effects of tumour-promoters lead to selective clonal expansion of initiated cells and are reversible, suggesting that the mechanism is epigenetic. These effects can be mediated through activation of the protein kinase C pathway. Resistance of initiated cells to phorbol ester-mediated terminal differentiation may relate to alteration in the expression of protein kinase C (via the initiating event). Evidence, to date, supports this molecular model for the differential effects of tumour promoters between normal and initiated cells. Phorbol esters produce different effects in different cell types. This may be explained by the expression of different classes of protein kinase C receptors among cell types. In addition, multiple protein kinase C genes and mRNA species have been identified in mammalian tissues and different rodent strains vary in their sensitivity to promoting agents.

Diversity in the elements that comprise the complex protein activation cascade likely are differentially expressed among the population, either because polymorphisms or differential exposures to environmental agents and variation in individual response to tumour promoters could result. Because of their role in differentiation, the protein kinase C gene family represent a target for anticancer therapy.

## **The Tumour-Suppressor Genes and Oncogenes**

Activation of proto-oncogenes and loss of tumour-suppressor genes are genetic changes associated with carcinogenesis. The study of mechanisms by which chemical carcinogens cause these changes is an active area of interdisciplinary cancer research. It clearly is reasonable that chemical-DNA interactions and carcinogen-DNA adduct formation (either direct, in the case of polycyclic aromatic hydrocarbons or indirect, in the case of oxyradicals) lead to this type of genetic change in human cancer, because these changes have been detected in tumours with a recognised chemical etiology. Furthermore, both the recognition that carcinogen-DNA interaction is an important step in carcinogenesis and the results of short-term mutagenesis assays led to the conclusion

that chemically induced DNA damage is an early step in the carcinogenic process.

Proto-oncogenes are normal cellular genes that control cell growth (i.e., proliferation), specialisation (i.e., differentiation) and death (i.e., apoptosis). Almost all proto-oncogenes encode a protein component of the signal transduction cascade. This integrated, multi-process system is responsible for the smooth orderly and specific transmission of extracellular signals to the nucleus and this process regulates gene transcription with respect to replication. When proto-oncogenes are activated, they are termed oncogenes. Oncogenes exert a positive driving force for cell growth by their failure to desist in response to the absence of stimulation. The discovery of oncogenes, their role in cell transformation and the realisation that these genes arise from the activation of normal cellular genes (proto-oncogenes) is also discussed. That there are several possible mechanisms by which proto-oncogenes may be activated should be emphasised. These are overexpression of the gene product, leading to an increased concentration of the protein (dosage hypothesis), expression of the gene at an inappropriate time or context, which could occur because of a mutation in the regulatory region of the gene (unscheduled gene expression), expression of a proto-oncogene in an inappropriate cell type and structural alteration of the gene product. The primary mechanisms by which chemicals cause oncogene activation are discussed later.

Activated ras genes predominate as the family of oncogenes to be isolated from solid tumours induced by chemicals in laboratory animals. Members of the ras gene family code for proteins of molecular weight 21,000, these proteins are membrane bound, have GTPase activity and form complexes with other proteins. The ras genes code for small G-proteins (guanine nucleotide binding) that exert a powerful proliferative response through the signal transduction cascade. They have been referred to as a molecular switch that, when mutated, freezes in the 'on' position. Activated ras binds to raf, a protein kinase and through this mechanism recruits other mitogen-activated protein kinases to cause cell proliferation. Disruption of this signalling pathway holds promise for future therapeutic strategies. The first direct evidence

of proto-oncogene activation by a chemical carcinogen was obtained from in vitro studies. A wild-type recombinant clone of the human Ha-ras gene (pEC) was modified with benzo[a]pyrene-diol-epoxide. The treated plasmid was then used to transfect NIH-3T3 cells, with the result that the transformed cell foci produced contained the same specific point mutations (in either codon 12 or 61) known to exist in activated ras genes isolated from human tumours including the bladder (pEJ).

In animal models of chemical carcinogenesis and surveys of different types of human tumours that arise from a variety of environmental exposures, ras mutations have been found. In rodents, polycyclic aromatic hydrocarbons (3-methylcholanthrene, 7,12-dimethylbenz [a] anthracene and benzo [a] pyrene) have been used repeatedly to produce both benign tumours and malignant carcinomas. A large proportion of these premalignant and malignant lesions have mutations in either the 12th or 61st codons. Similarly, treatment of rats with either 7,12-dimethylbenz[a]anthracene or N-methyl-N-nitrosourea resulted in the development of mammary carcinomas containing ras codon 12 or 61 mutations. These types of mutation also have been observed in mouse skin after initiation with 7,12-dimethylbenz[a]anthracene and tumour promotion with 12-O-tetradecanoylphorbol-13-acetate. Mutations in ras have been found in mouse liver after treatment with vinyl carbamate, hydroxydehydroestradiol or N-hydroxy-2-acetylaminofluorene. The same point mutations also have been found in murine thymic lymphomas after treatment with N-methyl-N-nitrosourea or radiation and in other rodent skin models after treatment with methylmethanesulphonate,  $\alpha$ -propiolactone, dimethylcarbamylic chloride or N-methyl-N9-nitro-N-nitrosoguanidine.

These data indicate that chemical carcinogens may produce site-specific mutations based, in part, on nucleoside selectivity of the ultimate carcinogen. Persistence of a specific mutation, however, also depends on the amino acid substitution, in that the function of the mutant protein is altered to confer on the cell a clonal growth advantage. The types of mutations that are found in chemically activated ras genes



cause conformational changes that alter nucleotide binding to the p21 protein in such a way that the p21 GTPase activity is not reduced. Data support the hypothesis that ras activation is associated with malignant conversion as well as tumour initiation. Transfection of activated ras genes into benign papillomas that did not contain a constitutively activated ras gene caused malignant progression.

Similarly, malignant transformation occurred when human bronchial epithelial cells were transfected with an activated ras gene, as well as when both c-raf-1 and c-myc were overexpressed in immortalised bronchial epithelial cells. In addition, Ki-ras gene mutations are one of a number of changes that can arise either early or late in the development of colorectal carcinoma. These findings indicate that it is the accumulation of mutations and not necessarily the order in which they occur, that contributes to multi-stage carcinogenesis. Furthermore, the stage of carcinogenesis in which each mutation occurs is not necessarily fixed. It appears that in the model for human colorectal carcinoma, ras mutations most often occur during malignant conversion but can be an early event (i.e., tumour initiation), in the rodent skin models, ras mutations appear to be primarily a tumour-initiating event. These differences may reflect the type of exposure, both in terms of chemical class and chronic versus acute exposure or they may be a function of tissue type.

Loss of the function of genes that may suppress the tumour phenotype, was considered as a theoretic possibility in regard to retinoblastoma more than 20 years ago. Firm experimental evidence for the existence of tumour suppressor genes was provided by analysis of the molecular genetics of pediatric tumours (retinoblastoma, Wilms' tumour, rhabdomyosarcoma and bilateral acoustic neurofibromatosis). Examination of DNA restriction fragment length polymorphisms by Southern hybridisation shows the loss of a restriction fragment from the tumour of a constitutive heterozygote, if that genetic locus has been affected by certain mutational events (deletion, translocation, nondisjunction, mitotic recombination). This type of genetic analysis is termed loss of heterozygosity (LOH). In fact, in pediatric tumours, studies that showed loss of the normal allele and duplication of the

inherited, defective allele provided the first proof of mitotic recombination in humans.

Loss of a tumour suppressor gene is generally characterised by a mutation in one copy of the gene and loss of the homologous copy. Examples of genes with these characteristics have been located on specific chromosomes: the retinoblastoma gene, the Wilms' tumour gene, the p53 gene, the von Hippel-Lindau disease gene, the neurofibromatosis genes, the adenopolyposis coli gene, the fragile histidine triad (fhit) and the p16 gene (9p21). Concerning tumour suppressor genes, most available data are derived from studies of p53. Point mutations in the p53 gene that give rise to amino acid changes or chain termination are observed in approximately 50% of human cancers and are frequent in lung as well as colon cancer.

The role of tumour suppressor genes (i.e., p53 in homeostasis) is to prevent tissue overgrowth, nullify cells with damaged genomes and metastasis. These controlling functions, even in the presence of already severely damaged cells that are being driven by activated proto-oncogenes (oncogenes), may be thwarted by a fully functional p53 protein. It is of further importance to recognise that the p53 function may be compromised by viral infection (e.g., human papillomavirus). The role of p53 in the life cycle of the cell is becoming increasingly well understood and the p53 protein has been shown to have broad functionality in cellular processes. These include cell cycle control, DNA repair, differentiation, genomic plasticity and apoptosis (i.e., programmed cell death).

Molecular analysis of the p53 gene may give clues to environmental etiology of cancer. It is implicit from the preceding text (DNA damage and repair) that the covalent binding of activated carcinogens to DNA is not random. Therefore, the formation of a particular DNA lesion, to some extent, may be deduced from the mutation that resulted. The p53 gene mutations in many human cancers could provide the clues. A dramatic example of this phenomenon is the previously mentioned codon 249 mutations, which is detected in almost all aflatoxin-related hepatocellular carcinomas. The striking nature of this association could

arise by two distinct mechanisms. First, the third base in codon 249 (AGG) may be unusually susceptible to activated aflatoxin B1 mutations. Indeed, it was discussed earlier that aflatoxin B1-8,9-oxide causes a promutagenic lesion by covalent binding to the N7-position of deoxyguanosine. Alternatively, cells bearing the codon 249 lesion may have a selective growth advantage. Evidence that a combination of these factors is responsible has been presented as well.

Another prominent example where circumstantial evidence points to specific molecular events is that of p53 mutations indicative of pyrimidine dimer formation in ultraviolet light-related skin cancers. In the case of tobacco smoking and lung cancer, G:C to T:A transversions indicate formation of adducts from activated bulky carcinogens (e.g., polycyclic aromatic hydrocarbons). However, we should be cautious in our interpretation because the two major confounding factors in this approach are that different carcinogens can lead to the formation of identical mutations and that most environmental chemical carcinogens are highly complex mixtures (e.g., tobacco smoke and diesel exhaust). These and other examples of mutational spectra at the p53 gene locus and others have been comprehensively reviewed.

The general mechanism for the loss of heterozygosity that occurs in tumours that have a familial origin may be different from that occurring in chemically induced cancer. Mitotic recombination is a common feature of pediatric neoplasms. The carcinogenic effects of the clastogens found in cigarette smoke, however, appear to be mediated, in part, more typically through chromosomal deletions. These deletions are primarily terminal, but they are to a lesser extent interstitial. Furthermore, given the complexity of tobacco smoke (a mixture of mutagens, carcinogens and promoters), these and other mutations likely result from both direct (adduct formation) and indirect (oxyradical formation) damage to DNA. Determination of these types of disease-associated mutational spectra, which include both oncogenes and tumour suppressor genes, eventually may be useful in defining causal chemical exposure.

## Clonal Evolution

The question of tumour clonality has been addressed primarily by examination of genetic markers. In particular, cytogenetic techniques for leukemia and LOH for cancer of the head and neck and colon cancer. In chronic myelogenous leukemia, the early disease phase is characterised by a single reciprocal translocation,  $t(9;22)$ , called the Philadelphia chromosome. This genetic change activates the *c-abl* proto-oncogene through the formation of a hybrid gene of *c-abl* with the break-point cluster region. The resulting gene product has elevated tyrosine kinase activity. The later stages of chronic myeloid leukemia are typified by overgrowth of one or more subclones that have additional karyotypic alterations.

In colorectal tumourigenesis, a paradigm has been developed in which accumulated alterations include at least one dominantly acting oncogene and several tumour suppressor genes. These same studies provided evidence for the progressive nature of genetic changes in carcinogenesis. LOH in these and other types of tumours always results in loss of one of a pair of restriction fragments, that is, the same allele (e.g., *p53*) in all the cells is evidence of clonality. The clonal origin of colorectal tumours in female patients with cancer has been more convincingly demonstrated by differential methylation. Inactivation of the X chromosome (by methylation) during embryogenesis is random, therefore, polyclonal female tissues develop with an approximately equal complement of inactivated maternal and paternal X chromosomes. If the tissues are monoclonal, the same inactive X chromosome should be present in all the cells. By using a DNA restriction fragment length polymorphism-based strategy, all the colorectal tumours were found to be monoclonal.

In cancer of the head and neck, the question of clonality as it pertains to field cancerisation was studied. Comparative molecular analysis found some paired tumours, arising in the same field, shared a common origin. These findings indicate that a single progenitor cell can replicate, expand and populate contiguous regions in the process of clonal expansion. However, the zonal limits of clonal expansion have not

yet been adequately determined. Genetic studies have found at least 95% of human tumours to be monoclonal in origin.

## Chemical and Viral Interactions

A distinction between viral and chemical carcinogenesis was made more than 50 years ago paving the way for the multi-stage theory of carcinogenesis. Certain viruses can integrate into the genome, depending on where they may activate the proto-oncogenes and/or inactivate the tumour suppressor genes. Because such viruses are able to act at every stage of carcinogenesis, it is reasonable that chemicals and viruses may have interactive effects. There now is good evidence from experiments with in vivo systems that viruses and chemicals also can interact in a synergistic manner. The causation of cancer by purely chemical means previously has been discussed and a number of studies clearly have demonstrated that certain forms of cancer have a viral etiology, including Burkitt's lymphoma and T-cell leukemia.

A number of human cancers now are considered to have both viral and chemical components to their etiology. Evidence exists for hepatitis B virus and aflatoxin B1 or alcoholic beverages in hepatocellular carcinoma, Epstein-Barr virus and N-nitrosamines in nasopharyngeal carcinoma and human papilloma virus and tobacco smoke components in cancers of the uterine cervix oral cavity and larynx. In essence chemicals can act as tumour promoters following tumour initiation by viral agents and viruses can act as promoters following chemical initiation. Cells that are pretreated with chemical carcinogens (benzo[a]pyrene, 4-nitroquinoline-N-oxide, 3-methylcholanthrene or thymidine analogues) have been shown to be morphologically transformed more easily by Simian virus 40 (SV40). Similarly, enhanced transformation by other viruses (adenovirus SA7, mutant adenovirus type 5 or herpes simplex virus type 2) also has been observed following pretreatment with several polycyclic aromatic hydrocarbons. In other cases, alkylating agents have been used (methylmethane sulphate) before infection with wild-type adenovirus 5 as a regimen to morphologically transform rat-embryo fibroblast cells in culture.

Human epithelial cells in vitro have proved to be more difficult for the study of chemicalviral interactions. Some reports exist where viruses (SV40 or Epstein-Barr) have been used first to immortalise the cells and chemicals (3-methylcholanthrene or N-acetoxy-2-acetylaminofluorene) have been used to cause neoplastic transformation of the immortalised cells. In Epstein-Barr virusimmortalised B-lymphocytes, however, N-methyl-N-nitrosoguanidine treatment failed to cause neoplastic transformation. Taken together, these studies may indicate that immortalisation, usually by the activation of telomerase, is required before malignant progression can occur and that more than one gene is involved. Although, it is difficult to assess the relevance of such an immortalisation step to human carcinogenesis in vivo, most human cancers contain an activated telomerase.

## The Implications of Molecular Epidemiology

The confluences of several important disciplines are responsible for molecular epidemiology. It encompasses the detection of carcinogenmacromolecular adducts (DNA as a direct genotoxic measure and protein as a surrogate), normal DNA sequence variants (heritable variations) and mutations in target genes (somatic changes). Therefore, these investigations use epidemiologic methods to investigate all aspects of geneenvironment interactions and risk assessment in human populations.

The biologically effective dose of a chemical carcinogen is governed by the amount that reaches a target tissue in a form that becomes activated in that tissue to a chemical species capable of causing lesions in DNA. Humans are most commonly exposed to complex mixtures of chemicals. Human carcinogen dosimetry at the molecular level requires sensitive and specific methods for carcinogenmacromolecular adduct quantitation. The low levels of adducts that are present in human DNA samples challenge the detection limits of conventional assay systems and complex mixtures of adducted materials confound simple assay systems.

The most commonly used methods that have been developed for

carcinogenDNA dosimetry in humans, specifically, the most commonly used techniques for adduct measurement, are P-nucleotide postlabeling, immunoassays, fluorescence spectroscopy, electrochemical conductance and gas chromatography/mass spectroscopy. Each of these techniques currently has its own advantages and limitations and within the framework of epidemiologic surveys, multiple corroborative end-point analyses seem to provide the most useful information. These methodologies, their application and their limitations are reviewed extensively elsewhere.

The most convincing example that correlates carcinogenDNA adduct levels with human environmental exposure and disease outcome is that of aflatoxin B1. Exfoliated aflatoxinN7 guanine adducts measured in urine samples, as well as aflatoxinalbumin adducts, were well correlated with both exposure and 6-hydroxycortisol levels, indicating a role for CYP3A4 in aflatoxin B1 activation. Importantly, the presence of aflatoxinN7 guanine adducts in urine was associated with liver cancer. Measurements of polycyclic aromatic hydrocarbonDNA adducts in the peripheral of occupationally exposed people have shown that this approach to human biomonitoring is feasible. Further research and development are required, however, to establish reliable methods. Studies to measure 4-aminobiphenylhemoglobin adducts have shown a doseresponse relationship between the extent of smoking, type of tobacco used and adduct levels.

Interindividual variations in cancer susceptibility and consequently meaningful human cancer risk assessment, involve determination of inherited host factors as well as exposure assessment. Most often these were determined by the use of indicator drugs (e.g., caffeine, debrisoquine, dextromethorphan, dapsone and isoniazid), however, increasingly metabolic polymorphisms are determined by direct genetic assays. This approach has further allowed for the determination of more diverse host factors, for which indicator drugs were not available. Thus, genetic indicators of propensity for carcinogen activation and detoxication, DNA repair capacity and cell cycle control ability are all features of molecular epidemiologic studies that are complementary to adduct studies because of the implications for biologically effective dose following exposure.

It is reasonable to suspect that cytochrome P-450 polymorphisms, involved in carcinogen activation and glutathione-S-transferases, UDPglucuronosyltransferases, sulfotransferases and N-actyltransferases, involved in both carcinogen activation and detoxication could explain variations in cancer susceptibility among the human population. It is fair to conclude that people absent protection of a functionally intact *gstml* gene are at increased risk of lung cancer from exposure to polycyclic aromatic hydrocarbons (PAHs) through tobacco smoke inhalation than those inheriting a functional variant of the gene.

Similarly, UDPglucuronosyltransferases (e.g., UGT1A1, UGT1A9, UGT2B7) have been implicated in cancer of the head and neck. Persons inheriting reduced activity variants of *nat1* and *nat2* genes, resulting in the slow acetylator phenotype, are at greater risk of aromatic amine induced bladder cancer, this may include persons exposed through tobacco smoke inhalation. Even though the inducible form of AHH (CYP1A1 and CYP1A2) has long been suspected of increasing cancer susceptibility in PAH-exposed persons, molecular epidemiology studies remain inconclusive. Similarly, studies of CYP2D6 metaboliser status and tobacco smoke related lung cancer is similarly confusing. However, analysis of multiple traits, e.g., CYP1A1 and GSTM1, in the same population may help to resolve these issues. Recent reviews confirm the current need for improved epidemiologic study design that integrates DNA adduct measures with indicators of metabolic capacity.

A large number of DNA repair genes have been described recently, but only few polymorphisms have yet been described. Nevertheless, molecular epidemiologic studies have provided evidence that genetic variation in these attributes can be a human cancer risk factor. The components of cell cycle control comprise genes that code for cell membrane receptors, intermediate messengers and transcription factors. An early molecular epidemiologic study that examined a potential link with an early intermediate messenger and cancer was that describing HRAS-1 rare alleles. For most cancers studied, ambiguous results have been recorded, but in the case of breast cancer, there have been 9 studies, all of which are positively correlated. Five are highly significant, 2 are borderline and 2 are not significant. Despite these



and other positive findings (lung and bladder), a molecular mechanism remains elusive. Polymorphisms in p53, brca-1 and waf-1 (p21) have also been studied. In general, no associations with increased cancer risk have been observed, however, several studies exist that have linked a specific p53 haplotype with breast cancer risk. Molecular characterisation of tumours, that is, molecular profiling, is emerging as an important tool that has both etiologic and clinical application.

During chemical carcinogenesis, the genome becomes altered and mutations accumulate. These mutations become evident in genes responsible for growth control and cellular homeostasis (including oncogenes, tumour suppressor genes and some DNA repair genes) because corruption of these functions is part of the carcinogenesis process. In respect to chemical carcinogenesis, the most studied genes are the Kirsten-ras (K-ras) oncogene and p53. K-ras is mutated in approximately 30% of lung adenocarcinomas and may prove to be an indicator of prognosis or a guide to treatment. The p53 tumour suppressor gene is mutated in many forms of human cancer and it is the most commonly mutated gene yet known (e.g., mutations in p53 are found in approximately 50% of lung cancers). Unlike ras gene mutations that are found in highly specific regions (codons 12, 13, 59 and 61), p53 mutations occur more widely. The reason for this is presumably because positive growth advantage conveys only with specific ras mutations and loss of p53 suppressor function can occur with less specificity. However, for some malignancies, p53 mutations have provided clues to cancer etiology. The best examples of this are mutations characteristic of aflatoxin exposure in liver cancer, radon or tobacco smoke in lung cancer and UV light in skin cancer. P53 is further distinguished from other genetic lesions, in that several possible mutant phenotypes can exist. Mutations may simply lead to the absence of p53, an inactive mutant protein may exist or the mutant might convey growth advantage. Several studies have investigated p53 expression and though its role in prognosis has not been clearly defined, it may be that it will provide a guide to treatment options. All mutant cancer genes are natural targets of gene- or gene-directed therapy. One notable success in this area is the use of Herceptin. Herceptin (or trastuzumab) is a humanised mouse monoclonal

antibody that has an affinity for HER-2, an epidermal growth factor receptor-related transmembrane receptor (p185). Herceptin preferentially mediates antibody-dependent cellular cytotoxicity in cancer cells that overexpress HER-2. The goal of molecular epidemiology is to identify risk factors for disease and outcome. Variations among humans in carcinogen biodistribution, metabolism, DNA adduct formation; DNA repair and potential response to tumour promoters have important implications in determining cancer risk. An increased understanding of the molecular basis of these differences and their connection with critical steps in carcinogenesis may assist in future predictions of disease risk before the clinical onset of disease. The facets of molecular epidemiology of human cancer risk are the assessment of carcinogen exposure and inherited and acquired host cancer-susceptibility factors. The interaction between these facets determines cancer risk. When combined with carcinogen bioassays in laboratory animals and classic epidemiology, molecular epidemiology can contribute to the four traditional aspects of cancer risk assessment: (1) hazard identification, (2) doseresponse assessment, (3) exposure assessment and (4) risk characterisation. Important bioethical considerations accompany the identification of high-risk individuals, these include autonomy, privacy, justice and equity. Benefits of the knowledge of risk for the individual may be offset by specific concerns relating to that individual's responsibility to family members and psychosocial anxiety regarding the genetic testing of children.

Therefore, the uncertainty of current individual risk assessments and the limited availability of genetic counselling services dictate caution. In addition, it is widely held that genetic testing should be restricted to those situations that are amenable to preventative or therapeutic intervention.

One facet of Molecular Epidemiology investigates geneenvironment interactions. Once the internalised chemical carcinogens are metabolised to reactive species that cause damage to DNA (carcinogenDNA adducts). The innate ability to repair DNA damage may reduce or ablate the overall damage burden. Alternatively, genetic changes (mutations, clastogenesis) may occur. Carcinogen metabolism

and DNA repair are categorisable genetic traits (host factors). DNA adducts (molecular dose) and mutational spectra are measures of exposure. Information from assays designed to investigate host factors and exposure measure can be used for human cancer risk assessment.

## **The Cell Proliferation**

The cancer cell differs from its normal counterpart in that it is aberrantly regulated. Cancer cells generally contain the full complement of biomolecules that are necessary for survival, proliferation, differentiation, cell death and expression of many cell-type-specific functions. Failure to regulate these functions properly, however, results in an altered phenotype and cancer.

Four cellular functions tend to be inappropriately regulated in a neoplasm. First, the normal constraints on cellular proliferation are ineffective. Second, the differentiation programme can be distorted. The tumour cells may be blocked at a particular stage of differentiation or they may differentiate into an inappropriate or abnormal cell type. Third, chromosomal and genetic organisation may be destabilised such that variant cells arise with high frequency. Some variants may have increased motility or enzyme production that permits invasion and metastases. Finally, the tightly regulated cell death program (apoptosis) may be dysregulated.

It is very important to understand the cancer cells and how their function becomes uncontrolled in cancer cells. It is also necessary to know how these are linked in the development of neoplasia.

## **Transmission of Biochemical Signals in Cancer**

### **Multicellular Organisms**

With the evolution of multicellular organisms, development of intercellular communication took place for such processes as embryonic development, tissue differentiation and as systemic responses to wounds and infections. These complex signalling networks

are in large part mediated by growth factors, cytokines and hormones. Such factors can influence cell proliferation in positive or negative ways, as well as induce a series of differentiated responses in appropriate target cells. The interaction of a growth factor with its receptor by specific binding in turn activates a cascade of intracellular biochemical events that is ultimately responsible for the biologic responses observed. Cytoplasmic molecules that mediate these responses have been termed second messengers. The eventual transmission of biochemical signals to the nucleus leads to effects on the expression of cassettes of genes involved in mitogenic and differentiation responses. The pathogenic expression of critical genes in growth factor signalling pathways can also contribute to altered cell growth associated with malignancy. The v-sis oncogene of simian sarcoma virus, which encodes a growth factor homologous to the B chain of human platelet-derived growth factor (PDGF-B), is the paradigm for such genes. The normal counterparts of other oncogenes have been shown to encode membrane-spanning growth factor receptors. Other genes that act early in intracellular pathways of growth factor signal transduction have been implicated as oncogenes as well. Present knowledge indicates that the constitutive activation of growth factor signalling pathways through genetic alterations affecting these genes contributes to the development and progression of most if not all human cancers. This chapter focuses on normal aspects of growth factor signalling, particularly those mediated by growth factor receptors possessing intrinsic protein tyrosine kinase activity. In addition, examples are provided where abnormalities in early steps in these pathways involving alterations in growth factor expression and/or receptor signalling have been implicated in the etiology of human malignancies. Finally, we will discuss how this knowledge may be useful in efforts to design new approaches toward therapeutic intervention with the malignant process. The limits of space preclude a discussion of several important families of ligands and their receptors. These include the cytokines and their receptors, which lack intrinsic tyrosine kinase activity but associate with cytoplasmic tyrosine kinases. Other ligand/receptor, families including the tumour necrosis factor, the T-cell receptor, the tumour growth factor (TGF), interleukins and Wnt receptor families,

will not be discussed. Finally, a group of small peptides, classified as neurotransmitters, has been shown under certain conditions to stimulate proliferation. Their receptors, which possess seven transmembrane domains, interact with heterotrimeric G proteins.

## The Oncogenes

An Oncogene is a gene that can cause to develop into a tumour cell, possibly resulting in cancer. A proto-Oncogene is a gene, which is involved in signal transduction and execution of mitogenic signals, usually through its protein product. Upon activation it becomes a tumour-inducing agent, an oncogene.

Since the early proposals of Boveri more than a century ago, multiple experimental evidences have confirmed that at the molecular level, cancer is due to lesions in the cellular DNA. First, it has been observed that a cancer cell transmits to its daughter cells the phenotypic features characterising the 'cancerous' state. Second, most of the recognised mutagenic compounds are also carcinogenic, having as a target cellular DNA. Finally, the karyotyping of several types of human tumours, particularly those belonging to the hematopoietic system, led to the identification of recurrent qualitative and numerical chromosomal aberrations, reflecting pathologic re-arrangements of the cellular genome. Taken together, these observations suggest that the molecular pathogenesis of human cancer is due to structural and/or functional alterations of specific genes whose normal function is to control cellular growth and differentiation or, in different terms, cell birth and cell death. The identification and characterisation of the genetic elements playing a role in the scenario of human cancer pathogenesis have been made possible by the development of DNA recombinant techniques during the last two decades. One milestone was the use of the DNA transfection technique that helped clarify the cellular origin of the 'viral oncogenes.' The latter were previously characterised as the specific genetic elements capable of conferring the tumorigenic properties to the RNA tumour viruses also known as retroviruses. Further, the transfection technique led to the identification of cellular transforming genes that do not have a viral counterpart.

Besides the source of their original identification, viral or cellular genome, these transforming genetic elements have been designated as proto-oncogene in their normal physiologic version and oncogene when altered in cancer. A second relevant experimental approach has regarded the identification and characterisation of clonal and recurrent cytogenetic abnormalities in cancer cells, especially those derived from the hematopoietic system. Several oncogenes have been thus defined by molecular cloning of the chromosomal breakpoints including translocations, inversions, etc. Additional oncogenes have been identified through the analysis of chromosomal regions anomalously stained (homogeneously staining regions [HSR]), representing gene amplification. Finally, the detection of chromosome deletions has been instrumental in the process of identification and cloning of a second class of cancer-associated genes, the tumour suppressors. Contrary to the oncogenes that are activated by dominant mutations and whose activity is to promote cell growth, tumour suppressors act in the normal cell as negative controllers of cell growth and are inactive in tumour cells. In general, therefore, the mutations inactivating tumour suppressor genes are of the recessive type. Recently, a third class of cancer-associated genes has been defined thanks to the analysis of tumours of a particular type, i.e., tumours in which an inherited mutated predisposing gene plays a significant role. These tumours include cancers in patients suffering from hereditary nonpolyposis colorectal cancer (HNPCC) syndromes.

The genes implicated in these tumours have been defined as mutator genes or genes involved in the DNA-mismatch repair process. Although not directly involved in the carcinogenesis process, these genes, when inactivated, expose the cells to a very high mutagenic load that eventually may involve the activation of oncogenes and the inactivation of tumour suppressors.

## **The Identification of Oncogenes**

Through the study of retroviruses the first oncogenes were discovered. RNA tumour viruses, whose genomes are reverse-transcribed into DNA in infected animal cells. During the course of infection, retroviral

DNA is inserted into the chromosomes of host cells. The integrated retroviral DNA, called the provirus, replicates along with the cellular DNA of the host. Transcription of the DNA provirus leads to the production of viral progeny that bud through the host cell membrane to infect other cells. Two categories of retroviruses are classified by their time course of tumour formation in experimental animals. Acutely transforming retroviruses can rapidly cause tumours within days after injection. These retroviruses can also transform cell cultures to the neoplastic phenotype. Chronic or weakly oncogenic retroviruses can cause tissue-specific tumours in susceptible strains of experimental animals after a latency period of many months. Although weakly oncogenic retroviruses can replicate in vitro, these viruses do not transform cells in culture.

Retroviral oncogenes are altered versions of host cellular proto-oncogenes that have been incorporated into the retroviral genome by recombination with host DNA, a process known as retroviral transduction. This surprising discovery was made through study of the Rous sarcoma virus (RSV). RSV is an acutely transforming retrovirus first isolated from a chicken sarcoma over 80 years ago by Payton Rous of RSV mutants in the early 1970s revealed that the transforming gene of RSV was not required for viral replication. Molecular hybridisation studies then showed that the RSV transforming gene (designated v-src) was homologous to a host cellular gene (c-src) that was widely conserved in eukaryotic species. Studies of many other acutely transforming retroviruses from fowl, rodent, feline and nonhuman primate species have led to the discovery of dozens of different retroviral oncogenes. In every case, these retroviral oncogenes are derived from normal cellular genes captured from the genome of the host. Viral oncogenes are responsible for the rapid tumour formation and efficient in vitro transformation activity characteristic of acutely transforming retroviruses. In contrast to acutely transforming retroviruses, weakly oncogenic retroviruses do not carry viral oncogenes. These retroviruses, which include mouse mammary tumour virus (MMTV) and various animal leukemia viruses, induce tumours by a process called insertional mutagenesis. This process results from integration of the DNA provirus into the host genome

in infected cells. In rare cells, the provirus inserts near a proto-oncogene. Expression of the proto-oncogene is then abnormally driven by the transcriptional regulatory elements contained within the long terminal repeats of the provirus. In these cases, proviral integration represents a mutagenic event that activates a proto-oncogene. Activation of the proto-oncogene then results in transformation of the cell, which can grow clonally into a tumour. The long latent period of tumour formation of weakly oncogenic retroviruses is therefore due to the rarity of the provirus insertional event that leads to tumour development from a single transformed cell. Insertional mutagenesis by weakly oncogenic retroviruses, first demonstrated in bursal lymphomas of chickens, frequently involves the same oncogenes (such as *myc*, *myb* and *erb B*) that are carried by acutely transforming retroviruses. In many cases, however, insertional mutagenesis has been used as a tool to identify new oncogenes, including *int-1*, *int-2*, *pim-1* and *lck*. The demonstration of activated proto-oncogenes in human tumours was first shown by the DNA-mediated transformation technique.

This technique, also called gene transfer or transfection assay, verifies the ability of donor DNA from a tumour to transform a recipient strain of rodent cells called NIH 3T3, an immortalised mouse cell line. This sensitive assay, which can detect the presence of single-copy oncogenes in a tumour sample, also enables the isolation of the transforming oncogene by molecular cloning techniques. After serial growth of the transformed NIH 3T3 cells, the human tumour oncogene can be cloned by its association with human repetitive DNA sequences. The first human oncogene isolated by the gene transfer technique was derived from a bladder carcinoma. Overall, approximately 20% of individual human tumours have been shown to induce transformation of NIH 3T3 cells in gene-transfer assays. The value of transfection assay was recently re-inforced by the laboratory of Robert Weinberg, which showed that the ectopic expression of the telomerase catalytic subunit (hTERT), in combination with the simian virus 40 large T product and a mutated oncogenic H-ras protein, resulted in the direct tumorigenic conversion of normal human epithelial and fibroblast cells. Many of the oncogenes identified



by gene-transfer studies are identical or closely related to those oncogenes transduced by retroviruses. Most prominent among these are members of the ras family that have been repeatedly isolated from various human tumours by gene transfer. A number of new oncogenes (such as neu, met and trk) have also been identified by the gene-transfer technique. In many cases, however, oncogenes identified by gene transfer were shown to be activated by re-arrangement during the experimental procedure and are not activated in the human tumours that served as the source of the donor DNA, as in the case of ret that was subsequently found genuinely re-arranged and activated in papillary thyroid carcinomas. Chromosomal translocations have served as guideposts for the discovery of many new oncogenes. Consistently recurring karyotypic abnormalities are found in many hematologic and solid tumours. These abnormalities include chromosomal re-arrangements as well as the gain or loss of whole chromosomes or chromosome segments. The first consistent karyotypic abnormality identified in a human neoplasm was a characteristic small chromosome in the cells of patients with chronic myelogenous leukemia (CML). Later identified as a derivative of chromosome 22, this abnormality was designated the Philadelphia chromosome, after its city of discovery. The application of chromosome banding techniques in the early 1970s enabled the precise cytogenetic characterisation of many chromosomal translocations in human leukemia, lymphoma and solid tumours.

The subsequent development of molecular cloning techniques then enabled the identification of proto-oncogenes at or near chromosomal breakpoints in various neoplasms. Some of these proto-oncogenes, such as myc and abl, had been previously identified as retroviral oncogenes. In general, however, the cloning of chromosomal breakpoints has served as a rich source of discovery of new oncogenes involved in human cancer.

## **Proto - Oncogenes Function**

Before mutation the protooncogene is found as a normal gene. An oncogene is dominant over a protooncogene. These genes are highly conserved throughout evolution and so are similar in sequence

between different species. Proto-oncogenes encode proteins that are involved in the control of cell growth. Alteration of the structure and/or expression of proto-oncogenes can activate them to become oncogenes capable of inducing in susceptible cells the neoplastic phenotype.

Oncogenes can be classified into five groups based on the functional and biochemical properties of protein products of their normal counterparts (proto-oncogenes).

These groups are (i) growth factors, (ii) growth factor receptors, (iii) signal transducers, (iv) transcription factors and (v) Programmed cell death regulators.

## **The Growth Factor Receptors**

The identification and study of altered growth factor receptors in experimental models of neoplasia have contributed much to our understanding of the normal regulation of cell proliferation. Some viral oncogenes are altered versions of normal growth factor receptors that possess intrinsic tyrosine kinase activity. Receptor tyrosine kinases, as these growth factor receptors are collectively known, have a characteristic protein structure consisting of three principal domains: (i) the extracellular ligand-binding domain, (ii) the transmembrane domain and (iii) the intracellular tyrosine kinase catalytic domain. Growth factor receptors are molecular machines that transmit information in a unidirectional fashion across the cell membrane. The binding of a growth factor to the extracellular ligand-binding domain of the receptor results in the activation of the intracellular tyrosine kinase catalytic domain. The recruitment and phosphorylation of specific cytoplasmic proteins by the activated receptor then trigger a series of biochemical events generally leading to cell division. Because of the role of growth factor receptors in the regulation of normal cell growth, it is not surprising that these receptors constitute an important class of proto-oncogenes. Examples include erb B, erb B-2, fms, kit, met, ret, ros and trk. Mutation or abnormal

expression of growth factor receptors can convert them into oncogenes. For example, deletion of the ligand-binding domain of erb B (the epidermal growth factor receptor) is thought to result in constitutive activation of the receptor in the absence of ligand binding. Point mutation in the tyrosine kinase domain or of the extracellular domain and deletion of intracellular regulatory domains can also result in the constitutive activation of receptor tyrosine kinases. Increased expression through gene amplification and abnormal expression in the wrong cell type are additional mechanisms through which growth factor receptors may be involved in neoplasia.

## Signal Transducers

Mitogenic signals are transmitted from growth factor receptors on the cell surface to the cell nucleus through a series of complex interlocking pathways collectively referred to as the signal transduction cascade. This relay of information is accomplished in part by the stepwise phosphorylation of interacting proteins in the cytosol. Signal transduction also involves guanine nucleotide-binding proteins and second messengers such as the adenylate cyclase system. The first retroviral oncogene discovered, *src*, was subsequently shown to be involved in signal transduction.

Many proto-oncogenes are members of signal transduction pathways. These consist of two main groups: nonreceptor protein kinases and guanosine triphosphate (GTP)-binding proteins. The nonreceptor protein kinases are subclassified into tyrosine kinases (e.g., *abl*, *lck* and *src*) and serine/threonine kinases (e.g., *raf-1*, *mos* and *pim-1*). GTP-binding proteins with intrinsic GTPase activity are subdivided into monomeric and heterotrimeric groups. Monomeric GTP-binding proteins are members of the important *ras* family of proto-oncogenes that includes H-*ras*, K-*ras* and N-*ras*. Heterotrimeric GTP-binding proteins (G proteins) implicated as proto-oncogenes currently include *gsp* and *gip*. Signal transducers are often converted to oncogenes by mutations that lead to their unregulated activity, which in turn leads to uncontrolled cellular proliferation.

## The Growth Factors

These are secreted polypeptides that function as extracellular signals to stimulate the proliferation of target cells. Appropriate target cells must possess a specific receptor in order to respond to a specific type of growth factor. A well-characterised example is platelet-derived growth factor (PDGF), an approximately 30-kd protein consisting of two polypeptide chains. PDGF is released from platelets during the process of blood coagulation. PDGF stimulates the proliferation of fibroblasts, a cell growth process that plays an important role in wound healing. Other well-characterised examples of growth factors include nerve growth factor, epidermal growth factor and fibroblast growth factor. The link between growth factors and retroviral oncogenes was revealed by study of the *sis* oncogene of simian sarcoma virus, a retrovirus first isolated from a monkey fibrosarcoma. Sequence analysis showed that *sis* encodes the beta chain of PDGF. This discovery established the principle that inappropriately expressed growth factors could function as oncogenes. Experiments demonstrated that the constitutive expression of the *sis* gene product (PDGF-b) was sufficient to cause neoplastic transformation of fibroblasts but not of cells that lacked the receptor for PDGF. Thus, transformation by *sis* requires interaction of the *sis* gene product with the PDGF receptor. The mechanism by which a growth factor affects the same cell that produces it is called autocrine stimulation. The constitutive expression of the *sis* gene product appears to cause neoplastic transformation by the mechanism of autocrine stimulation, resulting in self-sustained aberrant cell proliferation. This model, derived from experimental animal systems, has been recently demonstrated in a human tumour. Dermatofibrosarcoma protuberans (DP) is an infiltrative skin tumour that was demonstrated to present specific cytogenetic features: reciprocal translocation and supernumerary ring chromosomes, involving chromosomes 17 and 22. Molecular cloning of the breakpoints revealed a fusion between the collagen type Ia1 (*COL1A1*) gene and PDGF- gene. The fusion gene resulted in a deletion of PDGF-exon 1 and a constitutive release of this growth factor. Subsequent experiments of gene transfer of DPS genomic DNA into NIH 3T3

cells directly demonstrated the occurrence of an autocrine mechanism by the human re-arranged PDGF-b gene involving the activation of the endogenous PDGF receptor. Another example of a growth factor that can function as an oncogene is int-2, a member of the fibroblast growth factor family. Int-2 is sometimes activated in mouse mammary carcinomas by MMTV insertional mutagenesis.

## The Transcription Factors

These are nuclear proteins that regulate the expression of target genes or gene families. Transcriptional regulation is mediated by protein binding to specific DNA sequences or DNA structural motifs, usually located upstream of the target gene. Transcription factors often belong to multigene families that share common DNA-binding domains such as zinc fingers. The mechanism of action of transcription factors also involves binding to other proteins, sometimes in heterodimeric complexes with specific partners. Transcription factors are the final link in the signal transduction pathway that converts extracellular signals into modulated changes in gene expression. Many proto-oncogenes are transcription factors that were discovered through their retroviral homologues. Examples include *erb A*, *ets*, *fos*, *jun*, *myb* and *c-myc*. Together, *fos* and *jun* form the AP-1 transcription factor, which positively regulates a number of target genes whose expression leads to cell division. *Erb A* is the receptor for the T3 thyroid hormone, triiodothyronine. Proto-oncogenes that function as transcription factors are often activated by chromosomal translocations in hematologic and solid neoplasms. In certain types of sarcomas, chromosomal translocations cause the formation of fusion proteins involving the association of *EWS* gene with various partners and resulting in an aberrant tumour-associated transcriptional activity. Interestingly, a role of the adenovirus *E1A* gene in promoting the formation of fusion transcript flaws in normal human fibroblasts was recently reported. An important example of a proto-oncogene with a transcriptional activity in human hematologic tumours is the *c-myc* gene, which helps to control the expression of genes leading to cell proliferation. The *c-myc* gene is frequently activated by chromosomal translocations in human leukemia and lymphoma.

## Programmed Cell Death

Generally normal tissues exhibit a regulated balance between cell proliferation and cell death. Programed cell death is an important component in the processes of normal embryogenesis and organ development. A distinctive type of programed cell death, called apoptosis, has been described for mature tissues. This process is characterised morphologically by blebbing of the plasma membrane, volume contraction, condensation of the cell nucleus and cleavage of genomic DNA by endogenous nucleases into nucleosome-sized fragments. Apoptosis can be triggered in mature cells by external stimuli such as steroids and radiation exposure. Studies of cancer cells have shown that both uncontrolled cell proliferation and failure to undergo programed cell death can contribute to neoplasia and insensitivity to anticancer treatments. The only proto-oncogene thus far shown to regulate programmed cell death is bcl-2. Bcl-2 was discovered by the study of chromosomal translocations in human lymphoma. Experimental studies show that bcl-2 activation inhibits programed cell death in lymphoid cell populations. The dominant mode of action of activated bcl-2 classifies it as an oncogene. The bcl-2 gene encodes a protein localised to the inner mitochondrial membrane, endoplasmic reticulum and nuclear membrane. The mechanism of action of the bcl-2 protein has not been fully elucidated, but studies indicate that it functions in part as an antioxidant that inhibits lipid peroxidation of cell membranes. The normal function of bcl-2 requires interaction with other proteins, such as bax, also thought to be involved in the regulation of programed cell death. It is unlikely that bcl-2 is the only apoptosis gene involved in neoplasia although additional proto-oncogenes await identification.

## The Oncogene Activation Mechanism

A genetic change to cellular proto-oncogenes is the main activation of oncogenes. The consequence of these genetic alterations is to confer a growth advantage to the cell. Three genetic mechanisms activate oncogenes in human neoplasms: (i) mutation, (ii) gene amplification

and (iii) chromosome re-arrangements. These mechanisms result in either an alteration of proto-oncogene structure or an increase in proto-oncogene expression. Because neoplasia is a multistep process, more than one of these mechanisms often contributes to the genesis of human tumours by altering a number of cancer-associated genes. Full expression of the neoplastic phenotype, including the capacity for metastasis, usually involves a combination of proto-oncogene activation and tumour suppressor gene loss or inactivation.

## **Somatic Mutation**

Mutations are permanent and sometimes transmissible changes to the genetic material. It can be caused by copying errors in the genetic material during cell division and by exposure to radiation, chemicals or viruses or can occur deliberately under cellular control by the process such as meiosis or by per mutation. In multi cellular organisms, mutations can be subdivided into germline mutations which can be passed on to progeny and somatic mutations, which often lead to the malfunction or death of a cell and can cause cancer. Mutations activate proto-oncogenes through structural alterations in their encoded proteins. These alterations, which usually involve critical protein regulatory regions, often lead to the uncontrolled, continuous activity of the mutated protein. Various types of mutations, such as base substitutions, deletions and insertions, are capable of activating proto-oncogenes. Retroviral oncogenes, for example, often have deletions that contribute to their activation. Examples include deletions in the amino-terminal ligand-binding domains of the erb B, kit, ros, met and trk oncogenes. In human tumours, however, most characterised oncogenic mutations are base substitutions (point mutations) that change a single amino acid within the protein. Point mutations are frequently detected in the ras family of proto-oncogenes (K-ras, H-ras and N-ras).

It has been estimated that as many as 15 to 20% of unselected human tumours may contain a ras mutation. Mutations in K-ras predominate in carcinomas. Studies have found K-ras mutations in about 30% of lung adenocarcinomas, 50% of colon carcinomas and 90% of

carcinomas of the pancreas. N-ras mutations are preferentially found in hematologic malignancies, with up to a 25% incidence in acute myeloid leukemias and myelodysplastic syndromes. The majority of thyroid carcinomas have been found to have ras mutations distributed among K-ras, H-ras and N-ras, without preference for a single ras family member but showing an association with the follicular type of differentiated thyroid carcinomas. The majority of ras mutations involve codon 12 of the gene, with a smaller number involving other regions such as codons 13 or 61. Ras mutations in human tumours have been linked to carcinogen exposure. The consequence of ras mutations is the constitutive activation of the signal-transducing function of the ras protein. Another significant example of activating point mutations is represented by those affecting the ret proto-oncogene in multiple endocrine neoplasia type 2A syndrome (MEN2A). Germline point mutations affecting one of the cysteines located in the juxtamembrane domain of the ret receptor have been found to confer an oncogenic potential to the latter as a consequence of the ligand-independent activation of the tyrosine kinase activity of the receptor. Experimental evidences have pointed out that these cysteine residue-involving mutations promote ret homodimerisation via the formation of intermolecular disulfide bonding, most likely as a result of an unpaired number of cysteine residues.

## The Amplification of Gene

The Gene amplification refers to the expansion in copy number of a gene within the genome of a cell. Gene amplification was first discovered as a mechanism by which some tumour cell lines can acquire resistance to growth-inhibiting drugs. The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogeneous staining regions (HSRs). DMs are characteristic minichromosome structures without centromeres. HSRs are segments of chromosomes that lack the normal alternating pattern of light and dark staining bands. Both DMs and HSRs represent large regions of amplified genomic DNA containing up to several hundred



copies of a gene. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth.

The frequent observation of DMs and HSRs in human tumours suggested that the amplification of specific proto-oncogenes may be a common occurrence in neoplasia. Studies then demonstrated that three proto-oncogene families *myc*, *erb B* and *ras* are amplified in a significant number of human tumours. About 20 to 30% of breast and ovarian cancers show *c-myc* amplification and an approximately equal frequency of *c-myc* amplification is found in some types of squamous cell carcinomas. *N-myc* was discovered as a new member of the *myc* proto-oncogene family through its amplification in neuroblastomas. Amplification of *N-myc* correlates strongly with advanced tumour stage in neuroblastoma, suggesting a role for this gene in tumour progression. *L-myc* was discovered through its amplification in small cell carcinoma of the lung, a neuroendocrine-derived tumour. Amplification of *erb B*, the epidermal growth factor receptor, is found in up to 50% of glioblastomas and in 10 to 20% of squamous carcinomas of the head and neck. Approximately 15 to 30% of breast and ovarian cancers have amplification of the *erbB-2* (*HER-2/neu*) gene. In breast cancer, *erbB-2* amplification correlates with advanced stage and poor prognosis. Members of the *ras* gene family, including *K-ras* and *N-ras*, are sporadically amplified in various carcinomas.

## Re-arrangements of Chromosomes

The different cancer cells contain marker chromosomes, which are made up of many chromosomal pieces. Recurring chromosomal re-arrangements are often detected in hematologic malignancies as well as in some solid tumours. These re-arrangements consist mainly of chromosomal translocations and, less frequently, chromosomal inversions. Chromosomal re-arrangements can lead to hematologic malignancy by two different mechanisms: (i) the transcriptional activation of proto-oncogenes or (ii) the creation of fusion genes. Transcriptional activation, sometimes referred to as gene activation, results from chromosomal re-arrangements that move a proto-

oncogene close to an immunoglobulin or T-cell receptor gene. Transcription of the proto-oncogene then falls under control of regulatory elements from the immunoglobulin or T-cell receptor locus. This circumstance causes deregulation of proto-oncogene expression, which can then lead to neoplastic transformation of the cell. Fusion genes can be created by chromosomal re-arrangements when the chromosomal breakpoints fall within the loci of two different genes. The resultant juxtaposition of segments from two different genes gives rise to a composite structure consisting of the head of one gene and the tail of another gene. Fusion genes encode chimeric proteins with transforming activity. In general, both genes involved in the fusion contribute to the transforming potential of the chimeric oncoprotein. Mistakes in the physiologic re-arrangement of immunoglobulin or T-cell receptor genes are thought to give rise to many of the recurring chromosomal re-arrangements found in hematologic malignancy. In some cases, the same proto-oncogene is involved in several different translocations (i.e. c-myc, ewr and ret).

## Gene Activation

The t (8;14)(q24;q32) translocation, found in about 85% of cases of Burkitt's lymphoma, is a well-characterised example of the transcriptional activation of a proto-oncogene. This chromosomal re-arrangement places the c-myc gene, located at chromosome band 8q24, and under control of regulatory elements from the immunoglobulin heavy chain locus located at 14q32. The resulting transcriptional activation of c-myc, which encodes a nuclear protein involved in the regulation of cell proliferation, plays a critical role in the development of Burkitt's lymphoma. The c-myc gene is also activated in some cases of Burkitt's lymphoma by translocations involving immunoglobulin light chain genes. These are t (2;8) (p12;q24), involving the locus located at 2p12 and t(8;22) (q24;q11), involving the locus at 22q11. Although the position of the chromosomal breakpoints relative to the c-myc gene may vary considerably in individual cases of Burkitt's lymphoma, the consequence of the translocations is the same deregulation of c-myc

expression, leading to uncontrolled cellular proliferation.

In some cases of T-cell acute lymphoblastic leukemia (T-ALL), the c-myc gene is activated by the t(8;14)(q24;q11) translocation. In these cases, transcription of c-myc is placed under the control of regulatory elements within the T-cell receptor locus located at 14q11. In addition to c-myc, several proto-oncogenes that encode nuclear proteins are activated by various chromosomal translocations in T-ALL involving the T-cell receptor or locus. These include HOX11, TAL1, TAL2 and RBTN1/Tgt1. The proteins encoded by these genes are thought to function as transcription factors through DNA-binding and protein-protein interactions. Over expression or inappropriate expression of these proteins in T cells is thought to inhibit T-cell differentiation and lead to uncontrolled cellular proliferation. A number of other proto-oncogenes are also activated by chromosomal translocations in leukemia and lymphoma. In most follicular lymphomas and some large cell lymphomas, the bcl-2 gene (located at 18q21) is activated as a consequence of t(14;18)(q32;q21) translocations. Overexpression of the bcl-2 protein inhibits apoptosis, leading to an imbalance between lymphocyte proliferation and programmed cell death. Mantle cell lymphomas are characterised by the t(11;14)(q13;q32) translocation, which activates the cyclin d1 (bcl-1) gene located at 11q13. Cyclin d1 is a G1 cyclin involved in the normal regulation of the cell cycle. In some cases of t-cell chronic lymphocytic leukemia and prolymphocytic leukemia, the tcl-1 gene at 14q32.1 is activated by inversion or translocation involving chromosome 14. The tcl-1 gene product is a small cytoplasmic protein whose function is not yet known.

## The Mechanism of Gene Fusion

The first example of gene fusion was discovered through the cloning of the breakpoint of the Philadelphia chromosome in chronic myelogenous leukemia (CML). The t(9;22)(q34;q11) translocation in CML fuses the c-abl gene, normally located at 9q34, with the bcr gene at 22q11. The bcr/abl fusion, created on the der(22) chromosome, encodes a chimeric protein of 210 kD with increased

tyrosine kinase activity and abnormal cellular localisation. The precise mechanism by which the bcr/abl fusion protein contributes to the expansion of the neoplastic myeloid clone is not yet known. The t(9;22) translocation is also found in up to 20% of cases of acute lymphoblastic leukemia (ALL). In these cases, the breakpoint in the bcr gene differs somewhat from that found in CML, resulting in a 185-kD bcr/abl fusion protein. It is unclear at this time why the slightly smaller bcr/abl fusion protein leads to such a large difference in neoplastic phenotype.

In addition to c-abl, two other genes encoding tyrosine kinases are involved in distinct gene fusion events in hematologic malignancy. The t(2;5)(p23;q35) translocation in anaplastic large cell lymphomas fuses the NPM gene (5q35) with the ALK gene (2p23). ALK encodes a membrane-spanning tyrosine kinase similar to members of the insulin growth factor receptor family. The NPM protein is a nucleolar phosphoprotein involved in ribosome assembly. The NPM/ALK fusion creates a chimeric oncoprotein in which the ALK tyrosine kinase activity may be constitutively activated. The t(5;12)(q33;p13) translocation, characterised in a case of chronic myelomonocytic leukemia (CMML), fuses the tel gene (12p13) with the tyrosine kinase domain of the platelet-derived growth factor receptor b gene (PDGFR-b at 5q33). The tel gene is thought to encode a nuclear DNA-binding protein similar to those of the ets family of proto-oncogenes.

Gene fusions sometimes lead to the formation of chimeric transcription factors. The t(1;19)(q23;p13) translocation, found in childhood pre-B-cell ALL, fuses the E2A transcription factor gene (19p13) with the PBX1 homeodomain gene (1q23). The E2A/PBX1 fusion protein consists of the amino-terminal transactivation domain of the E2A protein and the DNA-binding homeodomain of the PBX1 protein. The t(15;17)(q22;q21) translocation in acute promyelocytic leukemia fuses the PML gene (15q22) with the RARA gene at 17q21. The PML protein contains a zinc-binding domain called a RING finger that may be involved in protein-protein interactions. RARA encodes the retinoic acid alpha-receptor protein, a member of the nuclear steroid/thyroid hormone receptor superfamily. Although

retinoic acid binding is retained in the fusion protein, the PML/RARA fusion protein may confer altered DNA-binding specificity to the RARA ligand complex. Leukemia patients with the PML/RARA gene fusion respond well to retinoid treatment. In these cases, treatment with all-trans retinoic acid induces differentiation of promyelocytic leukemia cells.

The ALL1 gene, located at chromosome band 11q23, is involved in approximately 5 to 10% of acute leukemia cases overall in children and adults. These include cases of ALL, acute myeloid leukemia (AML) and leukemias of mixed cell lineage. Among leukemia genes, ALL1 (also called MLL and HRX) is unique because it participates in fusions with a large number of different partner genes on the various chromosomes. Over 20 different reciprocal translocations involving the ALL1 gene at 11q23 have been reported, the most common of which are those involving chromosomes 4, 6, 9 and 19. In approximately 5% of cases of acute leukemia in adults, the ALL1 gene is fused with a portion of itself. This special type of gene fusion is called self-fusion. Self-fusion of the ALL1 gene, which is thought to occur through a somatic recombination mechanism, is found in high incidence in acute leukemias with trisomy 11 as a sole cytogenetic abnormality. The ALL1 gene encodes a large protein with DNA-binding motifs, a transactivation domain and a region with homology to the *Drosophila trithorax* protein (a regulator of homeotic gene expression). The various partners in ALL1 fusions encode a diverse group of proteins, some of which appear to be nuclear proteins with DNA-binding motifs. The ALL1 fusion protein consists of the aminoterminal of ALL1 and the carboxylterminus of one of a variety of fusion partners. It appears that the critical feature in all ALL1 fusions, including self-fusion, is the uncoupling of the ALL1 amino-terminal domains from the remainder of the ALL1 protein.

Solid tumours, especially sarcomas, sometimes have consistent chromosomal translocations that correlate with specific histologic types of tumours. In general, translocations in solid tumours result in gene fusions that encode chimeric oncoproteins. Studies thus far indicate that in sarcomas, the majority of genes fused by translocations encode

transcription factors. In myxoid liposarcomas, the t(12;16)(q13;p11) fuses the FUS (TLS) gene at 16p11 with the CHOP gene at 12q13. The FUS protein contains a transactivation domain that is contributed to the FUS/CHOP fusion protein. The CHOP protein, which is a dominant inhibitor of transcription, contributes a protein-binding domain and a presumptive DNA-binding domain to the fusion. Despite knowledge of these structural features, the mechanism of action of the FUS/CHOP oncoprotein is not yet known. In Ewing's sarcoma, the t(11;22)(q24;q12) fuses the EWS gene at 22q12 with the FLI1 gene at 11q24. Like FUS, the EWS protein contains three glycine-rich segments and an RNA-binding domain. The FLI1 protein contains an ets-like DNA-binding domain. The EWS/FLI1 fusion protein combines a transactivation domain from EWS with the DNA-binding domain of FLI1. In alveolar rhabdomyosarcoma, the t(2;13)(q35;q14) fuses the PAX3 gene at 2q35 with the FKHR gene at 13q14. The PAX3 protein, a transcription factor that activates genes involved in development, is a paired-box homeodomain protein with two distinct DNA-binding domains. The FKHR protein encodes a conserved DNA-binding motif (the fork head domain) similar to that first identified in the *Drosophila* fork head homeotic gene. The PAX3/FKHR fusion protein is a chimeric transcription factor containing the PAX3 DNA-binding domains, a truncated fork head domain and the carboxy-terminal FKHR regions.

In dermatofibrosarcoma protuberans (DP), an infiltrating skin tumour, both a reciprocal translocation t(17;22)(q22;q13) and supernumerary ring chromosomes derived from the t(17;22) have been described.

While early successful studies in this field have been performed with lymphomas and leukemia, as we have discussed before, the first chromosomal abnormality in solid tumours to be characterised at the molecular level as a fusion protein was an inversion of chromosome 10 found in papillary thyroid carcinomas. In this tumour, two main recurrent structural changes have been described, including inv(10)(q11.2; q21.2), as the more frequent alteration and a t(10;17)(q11.2; q23). These two abnormalities represent the cytogenetic mechanisms which activate the proto-oncogene *ret* on

chromosome 10, forming the oncogenes RET/ptc1 and RET/ptc2, respectively. Alterations of chromosome 1 in the same tumour type have then been associated to the activation of NTRK1 (chromosome 1), an NGF receptor which, like RET, forms chimeric fusion oncogenic proteins in papillary thyroid carcinomas. A comparative analysis of the oncogenes originated from the activation of these two tyrosine kinase receptors has allowed the identification and characterisation of common cytogenetic and molecular mechanisms of their activation. In all cases, chromosomal re-arrangements fuse the tK portion of the two receptors to the 5'end of different genes that, due to their general effect, have been designated as 'activating genes.' In the majority of cases, the latter belong to the same chromosome where the related receptor is located, 10 for RET and 1 for NTRK1.

Furthermore, although functionally different, the various activating genes share the following three properties:

1. They are ubiquitously expressed.
2. They display domains demonstrated or predicted to be able to form dimers or multimers.
3. They translocate the tK-receptor-associated enzymatic activity from the membrane to the cytoplasm.

These characteristics can explain the mechanism(s) of oncogenic activation of ret and NTRK1 proto-oncogenes. In fact, following the fusion of their tK domain to activating gene (1) ret and NTRK1, whose tissue-specific expression is restricted to subsets of neural cells, become expressed in the epithelial thyroid cells; (2) their dimerisation triggers a constitutive, ligand-independent transautophosphorylation of the cytoplasmic domains and as a consequence, the latter can recruit SH2 and SH3 containing cytoplasmic effector proteins, such as Shc and Grb2 or phospholipase C gamma (PLC), thus inducing a constitutive mitogenic pathway; (3) the relocation in the cytoplasm of ret and NTRK1 enzymatic activity could allow their interaction with unusual substrates, perhaps modifying their functional properties.

In PTCs, the oncogenic activation of ret and NTRK1 proto-oncogenes following chromosomal re-arrangements occurring in breakpoint cluster regions of both proto-oncogenes could be defined as an ectopic, constitutive and topologically abnormal expression of their associated enzymatic (tK) activity.

### **Oncogene Amplification in Human Cancers**

Tumour type	Gene amplified	Percent
Neuroblastoma	MYCN	2025
Small cell lung cancer	MYC	1520
Glioblastoma	ERB B-1 (EGFR)	3350
Breast cancer	MYC	20
	ERB B-2 (EGFR2)	~20
	FGFR1	12
	FGFR2	12
	CCND1 (cyclin d1)	1520
Esophageal cancer	MYC	38
	CCND1 (cyclin d1)	25
Gastric cancer	K-RAS	10
	CCNE (cyclin e)	15
Hepatocellular cancer	CCND1 (cyclin d1)	13
Sarcoma	MDM2	1030
	CDK4	11
Cervical cancer	MYC	2550
Ovarian cancer	MYC	2030



	ERB B-2 (EGFR2)	1530
	AKT2	12
Head and neck cancer	MYC	710
	ERB B-1(EGFR)	10
	CCND1(cyclin d1)	~50
Colourectal cancer	MYB	1520
	H-RAS	29
	K-RAS	22
MYCN Correlation		
Stage at diagnosis (%)	MYCN amplification	Three-year survival Bening
ganglioneuromas	0/64 (0%)	100
Low stages	31/772 (4%)	90
Stage 4-S	15/190 (8%)	80
Advanced stages	612/1.974 (31%)	30
Total	658/3000 (22%)	50

## Neoplasia Initiation and Progression

Human neoplasia is a complex multistep process involving sequential alterations in proto-oncogenes (activation) and in tumour suppressor genes (inactivation). Statistical analysis of the age incidence of human solid tumours indicates that five or six independent mutational events may contribute to tumour formation. In human leukemias, only three or four mutational events may be necessary, presumably involving different genes.

The study of chemical carcinogenesis in animals provides a foundation for our understanding the multistep nature of cancer. In the mouse

model of skin carcinogenesis, tumour formation involves three phases, termed initiation, promotion and progression. Initiation of skin tumours can be induced by chemical mutagens such as 7,12-dimethylbenzanthracene (DMBA). After application of DMBA, the mouse skin appears normal. If the skin is then continuously treated with a promoter, such as the phorbol ester TPA, precancerous papillomas will form. Chemical promoters such as TPA stimulate growth but are not mutagenic substances. Over a period of months of continuous application of the promoting agent, some of the papillomas will progress to skin carcinomas. Treatment with DMBA or TPA alone does not cause skin cancer. Mouse papillomas initiated with DMBA usually have H-ras oncogenes with a specific mutation in codon 61 of the H-ras gene. The mouse skin tumour model indicates that initiation of papillomas is the result of mutation of the H-ras gene in individual skin cells by the chemical mutagen DMBA. For papillomas to appear on the skin, however, growth of mutated cells must be continuously stimulated by a promoting agent. Additional unidentified genetic changes must then occur for papillomas to progress to carcinoma.

Although a single oncogene is sufficient to cause tumour formation by some rapidly transforming retroviruses such as RSV, transformation by a single oncogene is not usually seen in experimental models of cancer. Other rapidly transforming retroviruses carry two different oncogenes that cooperate in producing the neoplastic phenotype. One well-characterised example of this type of cooperation is the avian erythroblastosis virus, which carries the *erb A* and *erb B* oncogenes. Cooperation between oncogenes can also be demonstrated by in vitro transformation studies using nonimmortalised cell lines. For example, studies have shown cooperation between the nuclear *myc* protein and the cytoplasmic-membrane-associated *ras* protein in the transformation of rat embryo fibroblasts. As previously reported, a cooperation between SV40 large T product and mutated H-ras gene also have been found necessary to transform normal human epithelial and fibroblast cells provided that they constitutively expressed the catalytic subunit of telomerase enzyme, indicating a more complex pattern in the neoplastic conversion of human cells.

Collaboration between two different general categories of oncogenes (e.g., nuclear and cytoplasmic) can often be demonstrated but is not strictly required for transformation. The production of transgenic mice expressing a single oncogene such as *myc* has also demonstrated that multiple genetic changes are necessary for tumour formation. These transgenic mice strains, in fact, generally show an increased incidence of neoplasia and the tumours that result frequently are clonal, implying that other events are necessary. The production of transgenic mice expressing a single oncogene such as *myc* has also demonstrated that multiple genetic changes are necessary for tumour formation.

Cytogenetic studies of the clonal evolution of human hematologic malignancies have provided much insight into the multiple steps involved in the initiation and progression of human tumours. The evolution of CML from chronic phase to acute leukemia is characterised by an accumulation of genetic changes seen in the karyotypes of the evolving malignant clones. The early chronic phase of CML is defined by the presence of a single Philadelphia chromosome. The formation of the *bcr/abl* gene fusion as a consequence of the t(9;22) translocation is thought to be the initiating event in CML. The biologic progression of CML to a more malignant phenotype corresponds with the appearance of additional cytogenetic abnormalities such as a second Philadelphia chromosome, isochromosome 17 or trisomy 8. These karyotypic changes are thought to reflect additional genetic changes involving an increase in oncogene dosage and loss or inactivation of tumour suppressor genes. Although the karyotypic changes in evolving CML are somewhat variable from patient to patient, the accumulation of genetic changes always correlates with progression from differentiated cells of low malignancy to undifferentiated cells of high malignancy.

The initiation and progression of human neoplasia involve the activation of oncogenes and the inactivation or loss of tumour suppressor genes. The mechanisms of oncogene activation and the time course of events, however, vary among different types of tumours. In hematologic malignancies, soft-tissue sarcomas and the papillary type of thyroid carcinomas, initiation of the malignant process predominantly involves chromosomal re-arrangements that activate

various oncogenes. Many of the chromosomal re-arrangements in leukemia and lymphoma are thought to result from errors in the physiologic process of immunoglobulin or T-cell receptor gene re-arrangement during normal B-cell and T-cell development. Late events in the progression of hematologic malignancies involve oncogene mutation, mainly of the ras family, inactivation of tumour suppressor genes such as p53 and sometimes additional chromosomal translocations.

In carcinomas such as colon and lung cancer, the initiation of neoplasia has been shown to involve oncogene and tumour suppressor gene mutations. These mutations are generally thought to result from chemical carcinogenesis, especially in the case of tobacco-related lung cancer, where a novel tumour suppressor gene (designated FHIT) has been found to be inactivated in the majority of cancers, particularly in those from smokers. In preneoplastic adenomas of the colon, the K-ras gene is often mutated. Progression of colon adenomas to invasive carcinoma frequently involves inactivation or loss of the DCC and p53 tumour suppressor genes. Gene amplification is often seen in the progression of some carcinomas and other types of tumours. Amplification of the erb B-2 oncogene may be a late event in the progression of breast cancer. Members of the myc oncogene family are frequently amplified in small cell carcinoma of the lung. As mentioned previously, amplification of N-myc strongly correlates with the progression and clinical stage of neuroblastoma. Although there is variability in the pathways of human tumour initiation and progression, studies of various types of malignancy have clearly confirmed the multistep nature of human cancer.

The initiation and progression of human neoplasia is a multistep process involving the accumulation of genetic changes in somatic cells. These genetic changes then consist in the activation of cooperating oncogenes and the inactivation of tumour suppressor genes, which both appear necessary for a complete neoplastic phenotype. Oncogenes are altered versions of normal cellular genes called proto-oncogenes. Proto-oncogenes are a diverse group of genes involved in the regulation of cell growth. The functions of proto-oncogenes include growth factors,

growth factor receptors, signal transducers, transcription factors and regulators of programmed cell death. Proto-oncogenes may be activated by mutation, chromosomal re-arrangement or gene amplification. Chromosomal re-arrangements that include translocations and inversions can activate proto-oncogenes by deregulation of their transcription or by gene fusion. Tumour suppressor genes, which also participate in the regulation of normal cell growth, are usually inactivated by point mutations or truncation of their protein sequence coupled with the loss of the normal allele.

The discovery of oncogenes represented a breakthrough for our understanding of the molecular and genetic basis of cancer. Oncogenes have also provided important knowledge concerning the regulation of normal cell proliferation, differentiation and programmed cell death. The identification of oncogene abnormalities has provided tools for the molecular diagnosis and monitoring of cancer. Most important, oncogenes represent potential targets for new types of cancer therapies. It is more than a hope that a new generation of chemotherapeutic agents directed at specific oncogene targets will be developed. The goal of these new drugs will be to kill cancer cells selectively while sparing normal cells. One promising approach entails using specific oncogene targets to trigger programmed cell death. One example of the accomplishment of such a goal is represented by the inhibition of the tumour-specific tyrosine kinase bcr/abl in CML. Our rapidly expanding knowledge of the molecular mechanisms of cancer holds great promise for the development of better-combined methods of cancer therapy in the near future.

## **New Health Products for Farm Animals**

To help prevent these losses, animal scientists are using biotechnology to develop an array of products to diagnose, treat and prevent disease in farm animals. An area where animal biotechnology has already had a profound impact is diagnostic testing. Many animal diseases are difficult to diagnose. A veterinarian often has to wait hours or days for laboratory results to confirm a diagnosis. In the meantime, the

veterinarian must either withhold treatment or risk using an inappropriate therapy.

Using biotechnology, scientists are developing fast, accurate diagnostic tests for many of the most common farm animal diseases. In some cases, a veterinarian can conduct the diagnostic tests right on the farm and immediately start the best therapy. Many of these new tests use monoclonal antibodies. Antibodies are proteins an animal's immune system produces in response to invasion by bacteria, viruses or parasites. Each kind of antibody binds specifically with a characteristic part of a particular kind of invader.

Scientists make monoclonal antibodies by fusing two kinds of cells. One is an immune system cell that produces antibodies, which bind to part of a particular disease-causing microbe, the other is a cancer cell. The cell that results from the fusion inherits from the immune system cell the ability to produce antibodies and from the cancer cell the ability to reproduce indefinitely. This new hybrid cell or hybridoma, acts as an antibody factory. The antibodies are called monoclonal because they are produced by identical 'clones' of the original hybridoma - all will bind with the identifying structure on the surface of the microbe in question. One application of monoclonal antibodies is as a test for brucellosis in cattle. This bacterial disease often causes cows to abort pregnancies and it can be spread to farmers and people who drink milk from infected cows. Vaccines protect pregnant animals from abortion, but vaccinated cows may become carriers of the disease. Conventional diagnostic tests cannot distinguish between the disease-causing microbe and the vaccine, which is made from the microbe. Thus, these tests cannot help the farmer determine which animals are carriers of the disease and which are not. But diagnostic tests using monoclonal antibodies are so specific; they distinguish between cattle that carry the disease-causing bacterium and those that carry only the vaccine. With the aid of these new tests, farmers can isolate carriers to prevent the spread of infection. Diagnostic tests are not just for diseases. A monoclonal antibody test to detect pregnancy in animals is so simple that a farmer can read the test right on the farm. Monoclonal antibodies are also used in a test to detect a disease called scours in piglets and

calves. Scours is a bacterial disease of newborn animals that causes diarrhoea. Many of the afflicted animals die of dehydration. By providing quick, accurate diagnosis, the monoclonal antibody test for scours permits immediate administration of therapy. The therapy for scours in calves is also based on monoclonal antibodies. Infected calves are fed monoclonal antibodies that coat the offending bacteria, preventing the microbes from causing diarrhoea. Some other therapies for animal diseases are based on recombinant DNA technology. Genetic engineering allows scientists to insert one or more new genes into an animal, plant or microbial cell. In developing treatments for an animal disease, for instance, an animal gene is inserted into a microbe or an animal cell that can be grown in a laboratory culture. The inserted gene is one that produces a natural disease-fighting protein. The microbes or animal cells become minute factories, producing large quantities of the therapeutic protein. For example, genetic engineering is used to produce interferons and interleukin-2, natural proteins that fight viruses. Veterinarians long have used antibiotics against bacterial diseases, but they have had few drugs to treat viral infections.

Interferons and interleukin-2 not only kill viruses directly, they also stimulate the animal's immune system, improving the immune system's ability to fight disease naturally. One target of research regarding interferons and interleukin-2 is shipping fever, a disease of cattle caused by several viruses infecting an animal simultaneously. Although the animal's immune system normally keeps the viruses in check, outbreaks of the disease may result from stress during transport to feedlots or market. Shipping fever costs the beef industry more than \$250 million a year. Injections of interferons or interleukin-2 before shipping may help to augment the cattle's natural immunity and prevent shipping fever.

## **Disease Preventive Vaccines**

In addition to producing new diagnostic tests and therapeutic proteins, animal scientists are using biotechnology to develop vaccines to prevent disease. A safe, effective vaccine for swine pseudorabies, a fatal herpes-virus, is already in use. An advantage of the swine pseudorabies

vaccine and other vaccines made through recombinant DNA technology is that they use only a small portion of the original microbe. Conventional vaccines use killed or weakened forms of the disease-causing microbe. Sometimes, when the microbes are not completely killed or sufficiently weakened, a conventional vaccine may cause the disease it was supposed to prevent. Disease-causing genes are not included in the genetically engineered vaccines. Therefore, the recombinant vaccines build up the body's immunity without the risk of causing disease. Another advantage of recombinant vaccines is the speed with which they can be developed. 'Conventional vaccine development may take 20 or 30 years, maybe even 100 years,' says Karen Jacobsen, University of Georgia College of Veterinary Medicine. 'This is one reason why we don't have vaccines for a lot of animal diseases today.'

Recombinant vaccines are being developed for foot-and-mouth disease, a highly contagious viral disease that infects cattle, sheep and other animals. Although this disease has been eradicated in North America, it still causes substantial livestock productivity losses elsewhere, particularly in developing countries. Infected herds must be slaughtered and contaminated ranches must be left idle for months to prevent new outbreaks of the disease.

Conventional foot-and-mouth vaccines are made by weakening the virus that causes the disease. These vaccines sometimes revert to the virulent state and they have caused outbreaks of the disease in Europe. The foot-and-mouth vaccine made with biotechnology cannot cause the disease because, as in other biotech vaccines, the disease-causing genes have been removed. The biotech vaccine does not need to be kept cold and, therefore, can be used in developing countries. In addition to being used in treating swine pseudorabies and foot-and-mouth disease, recombinant DNA technology is being used to develop vaccines against dozens of livestock and poultry diseases, such as scours and a variety of respiratory disorders. Some vaccines may be injected into eggs so that chickens are immunised before they hatch. Most vaccines protect against viral or bacterial infections, but using genetic engineering, researchers in New Zealand and Australia have developed



a vaccine against a parasitic disease. Farmers must destroy the meat from sheep infested with a species of tapeworm common in those two countries. The new vaccine, which protects sheep from the tapeworm, may save farmers millions of dollars annually.

## **The Nutritional Protein for Animals**

Natural proteins called somatotropins or growth hormones help animals convert feed into muscle or milk. Using recombinant DNA technology, scientists have been able to develop bacteria that produce commercial quantities of somatotropins. They have found that administering small additional amounts of these proteins to animals helps to increase the animals' efficiency of feed conversion, thereby saving the farmer money.

Porcine somatotropin (PST) may help improve human health, as well as lower the farmer's cost of production. PST improves feed efficiency in hogs by 15 to 20 percent. It also reduces fat deposition, allowing PST-treated hogs to provide consumers with leaner cuts of pork. The ability to produce lean pork has enormous implications for improving human health by reducing dietary fat and cholesterol.

Another growth protein, bovine somatotropin (BST), is being administered to make dairy cows more efficient, thereby lowering the dairy farmer's costs. Regular administration of small amounts of BST increases a cow's milk yield by 10 to 25 percent. Although feed intake also increases, this feed increase is proportionately less than the increase in milk production. Studies indicate that BST can improve the milk-to-feed ratio by 5 to 15 percent.

Extensive laboratory testing has shown that the meat and milk of treated animals contain no more PST or BST than the meat or milk from untreated animals. Other tests have shown that these proteins do not act on the human body and therefore, they are safe to eat. Nevertheless, the use of BST remains controversial. Testing is under way to make sure that animals are not adversely affected by treatment with somatotropins.

Another protein that can be administered to animals to increase feed efficiency is called growth hormone releasing factor (GHRF). While not a growth protein itself, GHRF causes the animal to increase production of growth proteins.

## **Animal Breed Improvement**

Long before anyone knew about genes or DNA, people were selectively breeding animals to produce desirable traits, such as strength, feed efficiency and disease resistance. Selective breeding is a crude technology, because each cross between two animals mixes hundreds of thousands of genes, both desirable and undesirable. Recombinant DNA technology is now making it possible for scientists to breed animals with great precision. One or more new genes can be inserted into an animal embryo without disturbing the rest of the animal's genes. The resulting animal with the new gene or genes is referred to as a transgenic animal.

Despite the differences in technique, the scientists using recombinant DNA have the same objectives as the selective breeder: improving efficiency, disease resistance and other desirable traits. But recombinant DNA provides a wider array of possible new traits, since recombinant techniques allow animal scientists to insert genes not only from the same or closely related species, but also from distantly related animals, plants and even microbes.

One possible application of gene transfer in animals is to develop dairy cows that produce more nutritious milk. Bottle feeding human infants with cow's milk is not an ideal substitute for breast feeding, because cow's milk differs from human milk in some important components. Scientists are looking for ways to transfer genes into dairy cows that will enable the cows to produce milk that more closely resemble human milk.

## **Parasitic Disease Control**

Parasitic disease continues to be a major health problem worldwide in humans and domestic animals. Applying biotechnology has led to

progress in prevention and control in many areas, particularly in regard to diagnosis.

Parasites, like most organisms, carry repetitive sequences in their genomes. These are sometimes tandem repeats (back-to-back in a chromosome) or they may be located at random throughout the genome. Often, repetitive sequences are intergenic and noncoding and are thus far more subject to evolution and change, increasing the likelihood that they will be genus or even species specific. Since detection of these sequences is not dependent upon gene expression, which may vary over time, repetitive sequence-based assays allow detection of the parasite at any life stage. The effectiveness of repetitive sequence-based polymerase chain reaction (PCR) methods of identification has been demonstrated for parasites of several clinically important genera (1). A PCR assay has also been developed to differentiate two closely related nematode species that are virtually indistinguishable by morphologic examination (2), as well as for diagnosis of *Toxoplasma* and *Cryptosporidium* infections (3).

Attempts to develop anti-parasite vaccines have met with multiple failures. The problems often result from lack of post-translational modification by currently used vector-host systems and the inability to regenerate the secondary and tertiary structure of the native immunogens. Antigen presentation in recombinant parasite vaccines has also been thought to be a problem resulting in failed efficacy. There is now some evidence to indicate that cloning of genes for antigens of interest to yield chimeric molecules expressed in the BCG strain of *Mycobacterium bovis* may provide improved antigen presentation to stimulate both humoral and cellular responses (4); however, it is unlikely that future applications of this technology will be simple. Nonetheless, work is proceeding on the application of biotechnology to the development of vaccines against parasites in most food animals.

A particularly striking exception to the marginal successes reported in other systems has been a recombinant vaccine for a parasitic helminth of sheep (5). This product yielded greater than 94% protection in experimental work and is now available as a commercial product.

A further possibility is the application of anti-idiotypic monoclonal

antibodies. When monoclonal antibodies are prepared against a monoclonal antibody, it is possible to obtain, in a very small number of cases, an antibody which mimics the original antigen. Results of work with *Cryptosporidium parvum* epsilon toxin suggest that animals immunised with anti-idiotypic monoclonals can be protected against toxin challenge.

## **The requirement of Transgenic Animals**

The following two requirements are very important;

1. Production efficiency of farm animals—the original area of interest among many university researchers.
2. Molecular farming: using livestock to produce medicines, nutraceuticals and tissues for transplant to humans.

‘Now most of the money is here (in molecular farming),’ said Pinkert, ‘and it’s mostly corporate money.’

## **Gene Addition by Random Insertion**

For basic research, Pinkert pointed out two different lines of approach possible with transgenic cells: gain of function or loss of function. To study gain of function merely requires addition of a new gene. Pinkert underscored the power of studying animals, not just cells. ‘Anytime we tried to alter one trait with one gene,’ he said, ‘in the end the influences of that gene on other traits was something we couldn’t know until we had the animals.’ During 1982-83 pioneering research featured transgenic mice given a copy of the gene for human growth hormone. In this case, the added gene inserted randomly in the mouse genome. It did not insert at the mouse gene for growth hormone. The addition of the gene for human growth hormone did not inactivate or ‘knock-out’ the genes for mouse growth hormone. A picture of two mice side by side, one with the extra gene and the other without, gave ‘visual impact of what this technology might do,’ Pinkert pointed out. Comparing the size of the mouse with the extra gene to the control was like comparing softball to a baseball.

But the control of the growth hormone gene's impact on the endocrine system was not sufficient. This brought home the issue of qualitative versus quantitative control of gene expression. Since then mouse researchers have been wrestling with that next layer of problems: how to control genes in time and tissue, 'temporally and spatially'?

## Inserting Gene by Homologous Recombination

To study loss of function of an existing gene is a bit trickier. It requires a system in which a modified or faulty version of a gene is inserted by homologous recombination. The new version inserts by trading places with the existing gene and 'knocks out' the existing version of the gene. In homologous recombination, the inserted gene is not randomly inserted into the genome, but rather it is targeted to insert at the site of the existing homologous gene.

'Long life cycles of farm animals slow genetic analysis,' said Pinkert. 'That's why researchers use smaller, faster-breeding animals such as mice as model systems to test their ideas and their DNA constructs.' Furthermore, the mouse is the only model system that combines homologous recombination with cloning to allow the study of modified genes in development of adult animals. Currently, in livestock homologous recombination is possible only with cells grown in tissue culture. This means a scientist can study the effect of knocked-out genes only on the physiology of the cell. The possible role of the gene in development from embryo to adult cannot be tested without a system of cloning: taking the original cell and growing an adult from it.

In 1997 the cloning of Dolly and engineering of Polly have combined transgenesis and cloning. This combination was an essential step in developing in livestock a system of homologous recombination to modify existing genes. Now researchers are eagerly working on making a system of homologous recombination to further test their ideas with farm animals.

Once such a system is available, animal scientists will be able to ask

questions about the roles of genes in development from embryo to adult using livestock, not just mice.

## **Commercialisation Issues**

Researchers first produced transgenic farm animals in 1985, yet as of 1997 Pinkert notes no products in the supermarket or at the pharmacy are produced using such animals. A major reason is the lack of a clear route to government approval. 'Some (regulatory issues) are still outstanding, affecting utility and acceptance,' he said. 'Environmental impact is a huge issue' especially for transgenic fish that would likely mingle with their wild relatives.

Commercialisation comes with a communication component. 'The reasons we put forth for transgenic animals influence public perception,' Pinkert warned. He ended by noting that the public perception of pioneering products such as BGH, PST and even FlavrSavr tomato has affected governmental and corporate approach to reviewing and commercialising products from transgenic animals.

## **Animal Cloning and Gene Transfer Timeline**

- 1891 first successful embryo transfer early 1900's in vitro embryo culture develops
- 1961 mouse embryo aggregation to produce chimeras
- 1966 first report of microinjection of mouse embryos
- 1973 foreign genes function after cell transfection
- 1974 development of teratocarcinoma cell transfer
- 1977 mRNA and DNA transferred to *Xenopus* eggs
- 1980 mRNA transferred into mammalian ova
- 1980-81 transgenic mice first documented
- 1981 transfer of ES cells derived from mouse embryos
- 1982 transgenic mice and a growth hormone phenotype

- 1983 tissue specific gene expression in transgenic mice
- 1985 transgenic domestic animals produced
- 1985 microinjection for transgenic pigs, sheep, rabbits, fish
- 1987 chimeric 'knock-out' mice described
- 1987 retrovirus mediated: transgenic chicken
- 1989 targeted DNA integration & germline chimeric mice
- 1989 microinjection for transgenic cattle (Russia)
- 1989 first sperm mediated reports in farm animals
- 1991 microinjection for transgenic goats first referred for publication
- 1993 germline chimeric mice produced using co-culture
- 1996 ES cells used for nuclear transfer: sheep
- 1997 somatic cells from adult sheep used for cloning by nuclear transfer (Dolly)

## **Different Methods of Gene Transfer**

1. Blastomere/embryo aggregation
2. Teratocarcinoma cell transfer
3. Retroviral infection
4. Microinjection of cells (oocytes) with DNA
5. Electrofusion
6. Nuclear transplantation
7. ES (embryonic stem) cell transfer
8. Sperm-mediated transfer
9. Particle bombardment ('gene gun')

## The Cryopreservation of Human Embryo

If there happens to be a surplus of embryos following selection for fresh transfer (usually between one to four embryos are transferred to the uterus), then embryos of sufficient quality may be considered for cryostorage. While embryos can be frozen at any preimplantation stage between one-cell (one day old) to the blastocyst stage (5-6 days old), in an attempt to minimise the freezing of excessive numbers of 'spare' embryos and to help pre-select the most potentially viable embryos, we generally choose to cryopreserve only at the blastocyst stage. In certain cases where all embryos need to be frozen without a fresh transfer (e.g., when a woman may be at risk from ovarian hyperstimulation that might be complicated by pregnancy), we generally freeze all embryos the day after egg collection at the one-cell stage.

Techniques of controlled-rate freezing are utilised that slowly cool embryos in cryoprotectant fluid ('anti-freeze' solution) from body temperature down to  $-196^{\circ}\text{C}$ , at which temperature they are stored in containers of liquid nitrogen called dewars. The embryos are actually contained within special indelibly labeled plastic vials or straws that are sealed prior to freezing. Once frozen, they are placed inside labeled tubes attached to aluminium canes and stored in numbered canisters within the liquid nitrogen dewar. Site and label designations are stored in three separate file systems to avoid confusion and misidentification



of cryopreserved embryos. When it comes to thaw the embryos, all available identifiers of the stored specimen must match and be confirmed before thawing commences. The embryos are thawed out at room temperature, which takes about one to two minutes. However, the most critical element of the thaw procedure is not the timing but the careful dilution of the cryoprotectant fluid to return the embryo to its favoured culture medium. This permits resumed growth and development in vitro. Once this is done, the embryo is assessed for cryodamage to determine if it is suitable for transfer. Experience has shown that if the embryo survives 50% or more intact, it is worthwhile to replace it. Embryos can accommodate such levels of cellular damage and still establish healthy pregnancies. All thawed embryos routinely undergo assisted hatching prior to transfer. The zona pellucida, which surrounds the embryo, has been shown to suffer a certain amount of hardening during cryopreservation. This can be overcome by artificially making an opening in the outer embryo shell.

Varying strategies may be applied according to how many and which embryos are thawed prior to transfer. It should be noted that not every couple undergoing IVF will need to worry about embryo freezing/thawing, since not every couple will have sufficiently large number of 'surplus' or non-transferred embryos available for freezing. Indeed, most couples have only one or two embryos frozen, so that all are thawed and any surviving are replaced. In the event that there are more than two or three embryos frozen, thawing is usually undertaken until two to three healthy appearing embryos are recovered. In some cases, this may mean that all the cryopreserved embryos are thawed, in others just two or three. There always remains a possibility that there may be no embryo survival after thaw occurs and no transfer is possible. If many early embryos are frozen, it is possible to thaw all of them and culture them for several days to allow selection of the best for transfer. When too many embryos are available for transfer then extra embryos of sufficient quality may be refrozen for later use. This course of action has produced healthy offspring, proving the efficacy of double freezing of embryos.

During a medication-prepared frozen/thawed embryo transfer cycle

as a patient, you will follow a treatment schedule using Synarel or Lupron, estrogen (pills, lozenge or patch) and progesterone (lozenge and/or suppository) in order to achieve appropriate endometrium (uterine wall lining) for embryo transfer. Following embryo transfer, estrogen and progesterone will be administered daily until the 7th to 8th week of pregnancy or until a negative pregnancy test.

The first pregnancy from a frozen/thawed human embryo was reported in 1983 and a birth from this source occurred the following year. Of 99,629 cases of Assisted Reproductive Technology (ART) in the United States in 2000, about 16% of cases (16,194) used frozen/thawed embryos. In 2000, live birth rates per thaw cycle were 18.3% versus 26.6% from fresh embryo transfer. At GRS, the ongoing pregnancy rate for IVF using frozen/thawed embryos is currently 52%.

## **Risk Factors**

### **Possible Advantages of Cryopreservation of Embryos include:**

- Reduction of the risk of triplets or quadruplets by cryopreservation of embryos exceeding an optimal number for transfer to an individual patient
- Possibly increasing pregnancy rates by replacing thawed embryos during spontaneous ovulatory cycles or cycles in which the estrogen and progesterone hormone levels do not exceed that which occurs naturally
- Possibly decreasing the number of stimulated ovary drug treatment cycles needed for the attainment of pregnancy

The primary concern with the use of cryopreservation techniques is the possible loss of embryos to cryoinjury, meaning some healthy embryos may not survive the stress of freezing & thawing. The exact number of embryos lost to cryoinjury varies, but it is very likely that freezing will cause loss of some embryos, perhaps as many as 25-50% of those cryostored. One interpretation of this is that cryopreservation

may even act as a 'selection gate' for the more viable embryos, though this has never been proven.

Another concern with cryopreservation is the potential risk of birth defects in children produced from frozen/thawed embryos. In the domestic animal industry, large-scale freezing and transfer of embryos has not resulted in increased birth defects. Studies to date on those human offspring arising from thawed embryos have not shown any significant increase in abnormalities when compared to pregnancy outcomes in the rest of the population. To optimise the likelihood of successful embryo cryopreservation at GRS, the mechanical processes of human embryo cryopreservation will be strictly controlled to minimise the chances of technical failure. A back-up freezing system is always available to decrease the risk of interruption in the freezing process, as well as generator back-up power in the event of a power outage. Individual embryos are placed in permanently labeled storage containers and identified according to origin, developmental stage and date frozen. Permanent records are kept at Georgia Reproductive Specialists for each individual's embryos. Liquid nitrogen dewars are connected to alarm systems to monitor the liquid nitrogen levels and prevent premature thawing. However, even with all these safeguards, the possibility of technical failure leading to loss of stored embryos following natural disaster cannot be totally and completely eliminated.

## **Cryostorage Period**

Along with all records of medical care and procedures, cryopreservation records are maintained in the strictest confidence. The disposition of any frozen embryos that are not transferred must be arranged in writing before cryopreservation. In the event that a successful pregnancy is established following a fresh or subsequent embryo thaw cycle, it will be at the discretion of the couple as to whether the remaining frozen embryos should continue to be cryostored or appropriately discarded or donated. This may include the option to donate the embryos for research or to other infertile couples for transfer.

Cryogenic preservation (storage below  $-100^{\circ}\text{C}$ ) of cell cultures is

widely used to maintain backups or reserves of cells without the associated effort and expense of feeding and caring for them.

The success of the freezing process depends on four important factors:

1. Proper handling and gentle harvesting of the cultures
2. Correct use of the cryoprotective agent
3. A controlled rate of freezing
4. Storage under proper cryogenic conditions

There are a wide variety of chemicals provide adequate cryoprotection. It is observed that dimethylsulfoxide (DMSO) and glycerol are the most convenient and widely used. DMSO is most often used at a final concentration of 5 to 15% (v/v). Some cell lines are adversely affected by prolonged contact with DMSO. This can be reduced or eliminated by adding the DMSO to the cell suspension at 4°C and removing it immediately upon thawing. If this does not help, lower the concentration then try glycerol. Glycerol is generally used at a final concentration of between 5 and 20% (v/v). Although less toxic to cells than DMSO, glycerol can cause osmotic problems, especially after thawing. Always add it at room temperature or above and remove slowly by dilution. High serum concentrations may also help cells survive freezing. Replacing standard medium-cryoprotectant mixtures with 95% serum and 5% DMSO may be superior for some overly sensitive cell lines, especially hybridomas.

The cooling rate used to freeze cultures must be just slow enough to allow the cells time to dehydrate, but fast enough to prevent excessive dehydration damage. A cooling rate of -1°C to -3°C per minute is satisfactory for most animal cell cultures. Larger cells or cells having less permeable membranes may require a slower freezing rate since their dehydration will take longer. The best way to control cooling rates is by using electronic programmable freezing units. Although expensive, they allow precise control of the freezing process, give very uniform and reproducible results and can freeze large numbers of vials. Most units are available with chart recorders for a permanent record of the cooling process. There are a variety of mechanical freezing units

that provide adequate control of the cooling rate and are relatively inexpensive. Some systems use racks designed to hold vials at predetermined depths in the neck of a liquid nitrogen freezer. The cooling rate is dependent on the total number of vials and the depth at which the rack is placed. Another approach uses an alcohol-filled container designed to slowly freeze vials placed in the system. The filled container is placed in an ultracold mechanical freezer where the alcohol acts as a bath to achieve more uniform heat transfer and cooling. After freezing overnight, the vials are removed from the canister and transferred to their final storage locations. Only freezers capable of continually maintaining temperature below  $-130^{\circ}\text{C}$  should be considered for long-term cryogenic storage. Although most liquid nitrogen cooled freezers and some specially designed mechanical freezers meet this requirement, most cell culture laboratories prefer liquid nitrogen freezers. The final choice is often based on the availability of a reliable supply of liquid nitrogen, the storage capacity required and the size of the budget. Liquid nitrogen freezers permit storage either in the vapour phase above the liquid at temperature between  $-140^{\circ}\text{C}$  and  $-180^{\circ}\text{C}$  or submerged in the liquid at a temperature below  $-196^{\circ}\text{C}$ . Using vapour phase storage greatly reduces the possibility of leaky vials or ampules exploding during removal.

## Cell Harvesting and Freezing

Treat the cells gently during harvesting since it is very difficult for cells damaged during harvesting to survive the additional damage that occurs during the freezing and thawing processes. You should be able to obtain up to  $1.5 \times 10^6$  cells from a near confluent T-75 flask (depending on cell type and degree of confluence). There should be enough cells to set up at least several vials at  $2 \times 10^5$  cells/vial.

1. Using a sterile pipette, remove and discard the old culture medium.
2. For a T-75 flask, rinse the cell monolayer with 5ml of calcium- and magnesium-free phosphate buffered saline (CMF-PBS) to remove all traces of foetal bovine serum.
3. Add 4 to 5mL of the trypsin solution (in CMF-PBS) to the flask

and allow cells to incubate for at least one minute. (Prewarming of the enzyme Corning offers a variety of cryogenic vial sizes and cap styles.

4. Check the progress of the enzyme treatment every few minutes on an inverted phase contrast microscope. Once all of the cells have rounded up, gently tap the flask to detach them from the plastic surface. Then add 5mL of growth medium to the cell suspension and, using the same pipette, vigorously wash any remaining cells from the bottom of the culture vessel.
5. Collect the suspended cells in a 15mL centrifuge tube and place on ice. Take a sample for counting and then spin at 100xg for 5 minutes to obtain a cell pellet. While the cells are spinning, do a viable cell count (with the trypan blue solution) and calculate the number of cells/mL and the total cell number.
6. Remove the supernatant from the centrifuged cells and resuspend the cell pellet in enough of the cryoprotective medium containing 10% DMSO (DMSO is most often used at a final concentration of 5 to 15%.) to give a final cell concentration of 1 to  $2 \times 10^6$  cells/mL. Although not directly toxic, DMSO is a very powerful solvent and is able to rapidly penetrate intact skin (leaving a fishy or garlicky taste in your mouth). As a result, there is a potential hazard associated with using this compound. It is very important to avoid contact with DMSO and dispose of any wastes containing DMSO properly.
7. Label the appropriate number of cryogenic vials with the cell line and the date. Then add 1.5 to 1.8mL of the DMSO containing cell suspension to each of the vials and seal.
8. Place the vials in the controlled rate freezer overnight. After 24 hours, the cells should be transferred to a liquid nitrogen freezer for permanent storage.
9. Record the appropriate information about the cells in your cell repository records. Fully detail in these records the culture's storage conditions, including all of the following information: culture identity, passage or population doubling level, date frozen, freezing

medium and method used, number of cells per vial, total number of vials initially frozen and the number remaining, their locations, their expected viability and results of all quality control tests performed (sterility, mycoplasma, species, karyotype, etc.). Additional culture information, especially its origin, history, growth parameters, special characteristics and applications, is also helpful and should be included whenever possible.

## Recovery and Cell Thawing

1. Using appropriate safety equipment, remove the vial from its storage location and carefully check both the label and storage record to ensure that it is the correct culture. Place the vessel in warm water, agitating gently until completely thawed. Rapid thawing (60 to 90 seconds at 37°C) provides the best recovery for most cell cultures; it reduces or prevents the formation of damaging ice crystals within cells during rehydration.
2. Since some cryoprotective agents may damage cells upon prolonged exposure, remove the agents as quickly and gently as possible. Several approaches are used depending on both the cryoprotective agents and characteristics of the cells:
  - a) Most cells recover normally if they have the cryoprotective agent removed by a medium change within 6 to 8 hours of thawing. Transfer the contents of the ampule or vial to a T-75 flask or other suitable vessel containing 15 to 20mL of culture medium and incubate normally. As soon as a majority of the cells have attached (usually 3 to 4 hours), remove the medium containing the now diluted cryoprotective agent and replace with fresh medium.
  - b) For cells that are sensitive to cryoprotective agents, removing the old medium is easily accomplished by gentle centrifugation. Transfer the contents of the vial or ampule to a 15mL centrifuge tube containing 10mL of fresh medium and spin for 5 minutes at 100xg. Discard the supernatant containing the cryoprotective agent and resuspend the cell pellet in fresh medium. Then transfer the cell suspension to a suitable culture vessel and incubate normally.

## **The Precautions**

Take special care to avoid contact with solutions containing DMSO. It is a very powerful polar solvent capable of rapidly penetrating intact skin and carrying in with it harmful contaminants such as carcinogens or toxins. Always use appropriate safety equipment when removing vials and ampules from liquid or vapour phase nitrogen freezers. A full-face shield, heavy gloves and lab coat are strongly recommended for protection against exploding vials or ampules. Corning strongly recommends that cryogenic vials always be stored in the vapour phase above the liquid nitrogen to reduce the possibility of the vials filling with liquid nitrogen during extended storage. Vials filled with liquid nitrogen may explode violently upon removal from the freezer.



## **The Different Applications of Biomedicals**

Transgenic manipulation for the production of human pharmaceuticals or transplant organs generally is not intended to cause changes that have physiologic effects on the animals themselves. Thus, although unexpected and undesirable phenotypic effects still can occur as a result of gene insertion or cloning technology, there generally are fewer potential animal welfare concerns associated with the production of transgenic farm animals for biomedical purposes than for agricultural purposes.

In pigs, there was evidence that the mammary tissue developed abnormally due to premature expression of the transgene and that the condition of the mammary gland might have caused lactation to be painful. Similar concerns arise in the case of blood-borne proteins and nutraceuticals if the products are produced at levels higher than the animal's normal physiologic levels.

### **Xenotransplantation**

In an attempt to prevent hyperacute rejection of pig organs by humans, pigs have been made transgenic for the expression of human complement proteins, which are involved in regulation of the immune response. No phenotypic abnormalities have been reported in pigs as a result of the expression of transgenes for these human proteins, although, since the pigs are produced by microinjection, there are the

usual inefficiencies in terms of the number of embryos microinjected relative to the number of transgenic animals born.

Research is underway to produce pigs that, in addition to carrying complement transgenes, have both copies of the gene encoding the enzyme that produces the antigen associated with rejection knocked out. The animal welfare implications of this genetic manipulation are unknown; however, the knockout, which causes changes in cellular carbohydrate structure, potentially could have deleterious physiologic effects on the animals (Dove, 2000) and also render them susceptible to infection with human viruses.

An important animal welfare concern related to xenotransplantation is the management and housing of pigs intended for use as organ sources. To minimise the potential for transmission of disease to human recipients, only specific pathogen free (SPF) pigs are used. SPF research animals are used in other contexts besides xenotransplantation, but their use raises several animal welfare issues. SPF pigs are born by hysterotomy or hysterectomy and then are reared in isolators for 14 days before being placed in the source herd or in the xenotransplantation facility. The natural weaning age for pigs is about eight weeks (three to four weeks in commercial practice) and piglets subjected to extremely early weaning like this are known to develop abnormal behaviours. Older pigs intended for testing or organ donation might be housed in social isolation in unusually barren (i.e., easily sanitisable) environments. Pigs are extremely social animals that, when given the opportunity, will spend considerable time each day foraging and that develop abnormal behaviours in confinement if not given the opportunity to root or build nests. In the United Kingdom, the Home Office Code of Practice (Her Majesty's Government, 2000) for organ-source pigs, while recognising the importance of maintaining biosecure facilities, nevertheless recommends that such pigs be housed in stable social groups and provided with environmental enrichment such as straw or other material suitable for manipulation. The Code requires justification if the animals' behavioural needs are to be compromised for a xenotransplantation protocol. There are no comparable standards for pigs intended for xenotransplantation in

the U.S. and the lack of standardisation of housing and care among U.S. facilities for these pigs is a source of concern. Although there are many forms of environmental enrichment available that are suitable for laboratory-housed pigs. Appropriate methods for organ-source pigs require development and evaluation.

## **The Other Biomedical Applications**

Farm animals might be genetically engineered for human biomedical applications other than xenotransplantation or the production of pharmaceuticals. Research is underway, for example, to produce a porcine model of cystic fibrosis and there already are farm animal models for retinal degeneration and neurodegenerative disease. As genetic engineering techniques for farm animals improve—particularly such that single base coding changes that are typical of many human genetic diseases can be introduced and the production and use of farm animal models becomes more economically feasible—it is likely that more models for disease research and toxicity testing will be developed. Discussion of the potential issues raised by these biomedical uses of farm animals is outside the scope of this report. However, the welfare implications will depend upon specific features of the model under study, including any unalleviated pain and suffering associated with the disease process itself, as well as the need for specialised husbandry and veterinary care requirements.

## **Farming Applications**

If genetic technology becomes more efficient and affordable, the primary farming applications of transgenesis and cloning likely will be to produce animals with increased growth, improved feed conversion, leaner meat, increased muscle mass, improved wool quality, improved disease resistance and increased reproductive potential. The technology also can be used to produce food of improved nutritional quality (nutraceuticals) or appeal.

The primary difference between traditional breeding and genetic engineering is the speed at which change typically occurs (although

naturally occurring mutations and recombination events also can cause rapid and dramatic change) and the single-gene nature of genetically engineered change. Traditional methods of selection are more likely to be subject to the checks and balances imposed by natural selection. Many related and apparently unrelated traits are correlated genetically; thus, selective breeding involves selecting for a whole phenotype rather than a single gene product. Because most production and behavioural traits in livestock are polygenic and our understanding of livestock genomes is poor, few traits can reliably and predictably be engineered or introduced by manipulating only one gene. For this reason, the production of a line of transgenics will require generations of selective breeding after the introduction of gene constructs into the founder generation to ensure that animals display the desired phenotype with few or no undesirable side effects.

However, it is clear that serious welfare problems also have resulted from traditional breeding techniques. Broiler chickens are a case in point. Breeding for increased growth has led to serious physical disabilities, including skeletal and cardiovascular weakness. A large percentage of broilers have gait abnormalities and these might be painful, making it difficult for the birds to walk to feeders and waterers. In addition, broiler hens must be severely feed restricted to prevent obesity and this feed restriction is associated with extreme hunger and a variety of behavioural problems, including problems with mating behaviour and hyper aggressiveness. Traditional selection of pigs for increased leanness has led to increased excitability during handling and and selection for high reproductive rates (either by shortening the interval between births or increasing the number of offspring born) or increased lactation also has led to welfare problems.

## **The Use of Genetically Modified Animals**

Although genetic modification is capable of generating welfare problems...no qualitative distinction can be made between genetic modification using modern genetic modification technology and modification produced by artificial selection. Several ethical frameworks for evaluating the animal welfare implications of

biotechnologies applied to animals have been proposed in an attempt to resolve this difficulty. For example, Rollin (1995) has proposed the use of the “principle of conservation”, which states that transgenic and cloned animals developed for agricultural uses should not be worse off than the founder animals or other livestock of the same species under similar housing and husbandry practices.

## The Welfare of Farm Animals

Genetic engineering certainly has the potential to improve the welfare of farm animals. Decreasing mortality and morbidity by increasing resistance to diseases or parasites or decreasing responses to ingestion of toxic plants, are obvious examples of welfare benefits and an area in which some transgenic research is focused. It also has been pointed out that transgenic animals might receive a higher standard of care than nontransgenic animals because of their greater economic value. Cloning could be used as a strategy for breed preservation to maintain genes that are important for adaptation and resistance to disease, but equally could result in a further narrowing of the gene pool, with possibly deleterious effects on animal health.

Improving disease resistance to decrease pain and suffering is an application of transgenic technology that has clear animal welfare benefits. But it should be stressed that animal welfare is multifaceted and this needs to be taken into account when assessing welfare impacts of the application of any technology—not just biotechnology. Important elements of animal welfare include freedom from disease, pain or distress; physiologic normality; and the opportunity to perform normal behaviours. While reducing disease clearly is beneficial, if this also permits animals to be confined more closely and thus decreases the opportunity for them to perform their normal behaviours, then the net effect on welfare could be negative.

Genetic engineering also could be used to deal with non-disease related welfare problems. It might be possible, for example, to engineer hens that produce only female offspring. This would eliminate the problems associated with surplus male chicks, which are killed at the hatchery.

The need for the so-called standard agricultural practices like castration and dehorning also could be reduced or eliminated by genetic engineering. Pigs are castrated to prevent boar taint in the meat, but this trait is strongly linked (genetically) and thus is amenable to genetic manipulation. Similarly, horns on cattle, which are removed because they cause injuries to humans and other cattle, are the result of a single gene that could be knocked out by genetic manipulation without affecting other desirable performance traits; genetically polled (hornless) breeds of cattle already are available and are produced by selective breeding.

In making assessments about the production of genetically engineered animals for farming, costs and benefits need to be weighed carefully. When expression of growth hormone is regulated appropriately in transgenic pigs, for example, the increases shown in growth and feed efficiency are modest and are similar to the increases that can be attained simply by injecting pigs with porcine growth hormone suggest that centuries of selection for growth and body composition might limit the ability of the pig to respond to additional growth hormone. Indeed, it is possible that we already have pushed some farm animals to the limits of productivity that are possible by using selective breeding and that further increases only will exacerbate the welfare problems that have arisen during selection.

The potential for reduction in genetic diversity in agricultural species also is posed by inappropriate application of certain biotechnologies. Transgenesis raises such concerns because each transgene integration event results in a genetically unique potential founder and only one founder normally is used to found a transgenic line. This can result in a profound genetic bottleneck unless genetic variability is restored to a production line by purposeful utilisation of a mating strategy involving backcrossing of the transgenic line to a large number of distinct, presumably nontransgenic, mates. The effects of cloning are more difficult to anticipate because competing processes are at issue. On the one hand, cloning by its nature produces identical copies of a particular individual, reducing genetic variability relative to what would have been transmitted via conventional breeding. On the other

hand, cloning makes it possible to save and utilise genetic variability that would not otherwise be available. For example, cloning could be employed to utilise the genetic resources from a steer that had proven to be a high performing individual. Cryopreserved cells could be utilised as donor material. Moreover, cloning is a tool that actually can be used to increase/maintain genetic variance in some situations quite independently of exploiting castrates. The tradeoff between the competing processes of loss and gain of genetic variance would be case-specific and it is hard to quantify in the absence of simulation modelling with validation from field observations. Whatever the mechanism causing it, loss of genetic diversity could limit the potential for future genetic improvement of breeds by selective breeding or biotechnologic approaches. Furthermore, disease could spread through susceptible populations more rapidly than through more genetically diverse populations.

A particularly serious concern that arises is susceptibility of species with low genetic diversity to infectious disease. Diversity of animal populations— particularly at major histocompatibility (MHC) loci— is a major factor preventing spread of disease. Different MHC types recognise different viral or bacterial epitopes encoded by pathogens for presentation to the immune system. In genetically diverse populations, pathogens can evade the immune response only if they adapt to each individual MHC type following transmission from one individual to another. The requirement for this evolutionary process provides a population of animals with significant protection against the spread of infection. Pathogens can evade host immune response more easily in genetically uniform populations. The consequences of the failure of immunorecognition are illustrated by the deadly epidemics of diseases—such as measles—spread by initial contact between Europeans and isolated New World populations that lacked adequate MHC diversity. Not only could enhanced susceptibility create significant risk for spread of “new” infectious diseases in “monocultures” of cloned or highly inbred animal populations, it also could create new reservoirs for the spread of zoonotic infections—like new strains of influenza—to humans.

## **Human Health and Genetic Engineering of Animals**

The genetic engineering of animals can lead to the fundamental changes in their relationship with one another (for example, regarding the genetic structure of their populations) and between them (or their products) and humans. There are currently major research efforts under way to develop the use of genetically engineered animals as sources for production of non-traditional materials for human use. Such uses can be divided into three major categories: biopharmaceuticals for animal and human use; life cells, tissues and organs for xenotransplantation; and raw materials for processing into the other useful and products. Several possible concerns that might in practice arise from the first to uses are discussed in the following sections.

### **Biopharmaceutical Production**

A large number of genes and coding useful protein products — hormones, blood proteins and others — have been introduced into domestic animals leading to their expression in milk, eggs or blood. Indeed, a recent report suggests that the same technology might be extended to the large-scale production of vaccines. Such “biopharming” applications have the potential to use well-established agricultural methods to produce large amounts of valuable products at relatively low expense as compared to fermentation. Although products of the



second and third applications will be novel, by and large, the process of production and the potential concerns are not likely to differ greatly from those seen in current practice, such as the use of animals or animal cell cultures to prepare live vaccines. Hormones or traditional products like meat, milk or leather. The standard products have to pass specific regulatory procedures and essentially the same regulatory framework should apply for products of both biopharming in standard technology as regards and issues such as purity of the final product, microbial contamination, levels of adventitious DNA and the like. Nevertheless, a few more specialised concerns arise.

## **Novel Pathogens Contamination**

Although there is no example yet of acquisition of any gene, including drug resistance markers, by bacterial flora living in a transgenic animal, spread of introduced genes remains a possibility. Of greater concern is the possibility for generation of potentially pathogenic viruses by recombination between sequences of the vector used to introduce a transgene and related, but not pathogenic, viruses that might be present in the same animal. These concerns are particularly acute for retroviral factors. Retroviruses appear to be efficient vehicles for inserting genes into many species, including chickens mice cattle and might provide significant advantages and success rate over pronuclear injection of DNA in the generation of transgenic offspring in many species, including chickens and pigs, there are endogenous proviruses (including the poor kind endogenous viruses (PERVs) discussed in a following section) that are competent for low-level replication in the host animal but have no apparent pathogenic consequences . Endogenous proviruses aren't DNA sequences that were derived from infection of germline cells with a retrovirus and that are transmitted from parent to progeny like any normal gene. Their attenuation relative to their exogenous, pathogenic, counterparts often is due to differences in transcriptional regulatory sequences in the long terminal repeats. Since many vectors, such as the widely-used ones derived from murine leukemia virus (MLV), have LTR sequences derived from pathogenic viruses, the presence of vector and endogenous proviruses and all cells

of the transgenic animal provides the potential for generating pathogenic recombinant viruses, by straightforward and well-understood mechanisms. Such concerns are particularly acute and chickens and pigs were infectious proviruses very similar in sequence to those used for vectors are known to be present. In mice, there is a well studied model in which recombination between benign endogenous proviruses or endogenous proviruses and infecting viruses early in the life of the animal can cause a high incidence of lymphoma (nearly 100 percent in some of strains) six months later. Given this example, it is reasonable to expect that viruses of much greater pathogenicity than existed before are likely to arise and animal when there is a possibility of recombination between vector and endogenous viral sequences

Some concerns arise with the use of vectors based on lentiviruses for introduction of genes Recombination of lentiviruses in circulation and domestic animal populations, such as FIV in cats and BIV in cattle, with vectors based on HIV is improbable to the large genetic distance between them. However vectors based on FIV and BIV is being developed and their use to introduce transgenes into the corresponding species would significantly increase the probability of generation of more pathogenic recombinants.

## Uses of Transgenic Animals for Pharmaceutical Production

### Examples of Products under Development

#### Theoretical Yield (g/yr of Species Raw Protein)

Chicken	250	monoclonal antibodies
		lysozyme
		growth hormone
		insulin
		human serum albumin

Rabbit	20	calcitonin
		superoxide dismutase
		erithropoiten
		growth hormone
		IL-2
Goat	4000	a-glucosidase
		antithrombin III
		tissues plasminogen activator
		monoclonal antibodies
		a-1-antitrypsin
Sheep	2500	growth hormone
		a-1-antitrypsin
		factor VIII
		factor IX
		fibrinogen
Cow	80,000	human serum albumin
		lactoferrin
		a-lactalbumin

## Confinement of Unwanted Animals

As long the animals do not contain the product of the induced gene, there might be no strong reason to believe that eating or using products from such animals would pose a threat to human health; however, the lack of regulatory oversight for such uses argues strongly for confinement measures. Although it has been stated that such animals will be too valuable to the owners to allow their misappropriation the fact that the products of interest usually are produced only by

lactation. Companies using biopharm animals are likely to seek approval for marketing food or rendered products from surplus animals and the regulatory agencies will need to be ready to deal with such requests. Of greater concern is the possibility that surplus animals (and their carcasses) might, inadvertence for theft, find their way into the food or rendering chain or be used for breeding, thus allowing uncontrolled spread of the transgene into the general population, creating the regulatory problem of dealing with a approved transgenes after their release into the food chain, a problem analogous to that posed by the appearance in food products of star link, a transgenic maize unapproved at the time for human consumption.

## Xenotransplantation

This technology differs from other uses of genetically engineered animals in that it has the potential to create something entirely new — permanent human-animal chimeras — in which cells of distantly-related species survived and function for long periods of time in the most intimate possible contact. Given its potential for alleviating human diseases due to irreversible tissue or organ failure and given the acute shortage of human organs for transplant, there are very active research programs underway, in both commercial and academic laboratories, to solve the significant immunological and physiological problems and thereby bring xenotransplantation into standard medical practice. This topic and the associated infectious concerns have been renewed in great detail elsewhere and only an overview will be given here.

## Applications of Xenotransplantation

Indication	Transplant	Status
Organ failure	take heart, kidney, liver, etc.	0
Acute liver failure	extracorporeal perfusion	1
Diabetes	pancreatic islets (or cells)	1
Parkinson's disease	Huntington's diseaseNeural Tissue	1

Focal epilepsy

Stroke

Burn	skin autograft	2
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Skin injury	(Co-cultured with mouse cells)
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Note:

0 = no successful experience

1 = some trials have been performed

2 = successful trials have been performed

At present, the only animal under serious consideration as a xenotransplantation is the pig (4 regulatory purposes, human cells cultured ex vivo with cells of any other animal, such as mouse alliance, also are considered to be xenotransplantation; DHHS, 2001; co-cultivation with mouse cell means has been used in preparation of some cultured skin grafts, as well as human stem cell lines. Well-known human primates, such as the baboon, would seem to have physiologic and immunogenetic advantages such as the lack of a hyperacute immune response, their scarcity as well as the difficulty of clearing them of adventitious infectious agents (as well as ethical concerns) renders them impractical for further consideration.

The field of xenotransplantation covers a great many procedures, ranging from implantation of single cells to treat Parkinson's disease; tissues, such as pancreatic islets for treatment of diabetes; extracorporeal use of contact organs, such as perfusion of patient blood through pig livers to provide short-term support in cases of liver failure; to transplantation of whole organs — heart, kidney, liver and so on. While whole-organ xenotransplantation remains far in the future, development of the simpler modalities is underway and hundreds of human subjects have received porcine cells or tissues as part of clinical trials in the United States, Russia, Israel and many European countries. Given the nature of infectious disease issues, regulatory concerns are not limited to the United States alone, but instead extend to the international health community as well.

The development of xenotransplantation as a part of clinical practice promises great benefits in terms of making possible essentially infinite supplies of replacement tissues and organs where severe shortages exist today. This development will naturally entail both great potential benefit as well as considerable risk to the study participant but such risk is not qualitatively different than the development of any other new medical procedure and will not be considered further. The principal concern is that the uniquely close relationship created between recipient and host will allow novel opportunities for transmission of infectious disease and possibly creation of new disease agents in the process. While the history of close contact between animals and pigs is a very long one and one would imagine that all possible transmission of infectious agents between the two species would already have been seen and thoroughly studied, it is possible that the "co-culture" endocrine milieu of a transplant would be qualitatively different in ways that would allow different outcomes. Two different types of agents are discussed separately.

## **Exogenous Infectious Agents**

As can be seen in the table given below the number of viral agents that are potential concern is very large. Not all of the viruses are on the list because of the potential to cause human disease others are sensitive indicators of breaks in biosecurity and so forth. In principal, since all of these agents are horizontally (one animal to another) or vertically (mother to offspring) transmitted, they can be eliminated by proper management—proper containment, vaccination, close monitoring, culling, birth by cesarean section, etc. In practice, elimination is going to prove a very difficult task, and there is lack of reliable assays for detecting many of them. Nevertheless, problems resulting from transmission of the exogenous infectious agents are not qualitatively different from the present situation with human donors (allotransplantation), where infection with agents transmitted with the transplanted organ (such as Epstein-Barr virus and cytomegalovirus) is a major problem. In fact, it is anticipated that reduction in the risk of acute morbidity and mortality resulting from

the transmission of infectious agents will be a significant benefit of xenotransplantation.

## Exogenous Paid Viruses of Concern in Xenotransplantation

Family	Species	Category
Picornaviridae	foot and mouth disease	
	enterovirus 1 Talfan/Teschen	2,5
	enterovirus (other serogroups)	5
	enterovirus swine vesicular disease	5
	human enteroviruses	1
	encephalomyocarditis	
	rhinovirus	
Caliciviridae	enteric calicivirus	1
	swine hepatitis E	1
Astroviridae	porcine astrovirus	5
Togaviridae	Western encephalitis	1
	Eastern encephalitis	1
	Venezuelan encephalitis	1
Getah		1
	chikungunya	1
Flaviviridae	Japanese B encephalitis	1
	Louping III/TBE complex	1
	Wesselbron disease	1
	Apoi	
	dengue fever	1
	West Nile fever	1

	classical swine fever (hog cholera)	5
	bovine viral diarrhoea	5
	border disease	5
Coronaviridae	transmissible gastroenteritis	4,5
	porcine respiratory coronavirus	4,5
	epidemic diarrhoea	4,5
	haemagglutinating encephalomyelitis	4,5
	porcine reproductive and respiratory disease syndrome	4,5
	porcine torovirus	5
Paramyxoviridae	murine parainfluenza virus	2
	type 1 (Sendai)	
	parainfluenza	2, 2
	parainfluenza	3, 2
	blue eye disease	5
	menangle	1
	nipah	1
Rhabdoviridae	vesicular stomatitis	1
	rabies	1
Bornaviridae	bornavirus	2,5
	orthomyxoviridae	
	influenza	1
	influenza c	5
bunyaviridae	akabane	1,5
	batai	1,5
	hantavirus	1,5



arenaviridae	lymphocytic choriomeningitis	1,5
	reoviridae ibaraki	5
	reovirus 1 to 3	2
	rotavirus a, b, c, e.	2
birmaviridae	porcine picobirnavirus	5
retroviridae	porcine endogenous	2
hepadnaviridae	hepatitis b	
circoviridae	porcine circovirus	5
parvoviridae	porcine parvovirus	4,5
papovaviridae	porcine polymavirus	3
porcine	genital papillomavirus	3,5
adenoviridae	porcine adenovirus serotypes	1 to 43
herpesviridae	pseudorabies	2
	porcine cytomeglovirus	
porcine	lymphotropic herpesvirus type	1 3
	porcine lymphotropic herpesvirus type	2 3
poxviridae	swinepox	5
	vaccinia	2
	cowpox	1,5
	orf/pseudocowpox	1,5*
desoxyviridae	African swine fever	5

Note:

1=zoonotic

2=replicates in human cells or weak evidence for zoonotic potential

3=might undergo abortive replication and possibly oncogenic

4=belongs to a family with evidence of frequent changes in host range or pathogenicity

5= undesirable as indicates a breakdown in biosecurity and or might compromise health of the pigs

\*= although the virus has not been recorded in pigs, it has been included for reasons such as its wide host range

## **Porcine Endogenous Retroviruses**

PERVs represent quite a different situation and level of concern, since they are inherited as part of the host genome and, therefore, cannot be removed easily from donor animals. All pigs contain multiple (around 50) PERV proviruses in their genome, at least several of which Inco to infectious virus. PERVs are gammaretroviruses, closely related to MLV, that can be classified into three subtypes, A, B and C, based on their envelope gene sequences. Subtypes A and B can infect many types of human cells and culture.

Subtype C is much less infectious for humans. Most breeds of pigs carry proviruses capable of yielding infectious virus of all three subtypes. Although most pigs carry about the same number of proviruses in their DNA, there is considerable diversity and location, implying that their insertion into the genome must have occurred relatively recently (on an evolutionary time scale). Based on extensive experience with related endogenous proviruses of mice, it is highly likely that the majority of proviruses contain some sort of genetic defect and that only a small number are responsible for release of infectious virus. Taken together with the polymorphism and presence or absence of specific proviruses, it might well be possible to breed animals lacking infectious proviruses for use as xenotransplant donors.

PERVs have not yet been shown to cause disease (or even viremia) and pigs or any other species in which they have been tested. Nor has their presence been detected (by PCR or serology) and over 150 human recipients of pig cells or tissues , although a low level of infection of recipients cells can be observed in immunodeficient mice transplanted with porcine islets of Langerhans. Nevertheless, given the release of

virus infectious for human cells by many types of pig cell, the close similarity of these viruses to viruses known to cause cancer, immunodeficiency and other diseases in mice and cats, the well-known adaptability and variability of retroviruses and the example of the rapid worldwide spread of HIV and AIDS, there is serious concern that the novel association between pig and human tissues might create novel evolutionary opportunities for the virus, leading to the appearance of a new pathogen. Although such a pathogen could have serious long-term adverse consequences to the transplant recipient, this issue is not an area of concern, since it is far away at by the potential benefit of the transplant. The real issue of concern is that the xenotransplant setting might prove the opportunity for the virus to evolve into a pathogen which can also be transmitted from one individual to another efficiently enough to create a new epidemic disease.

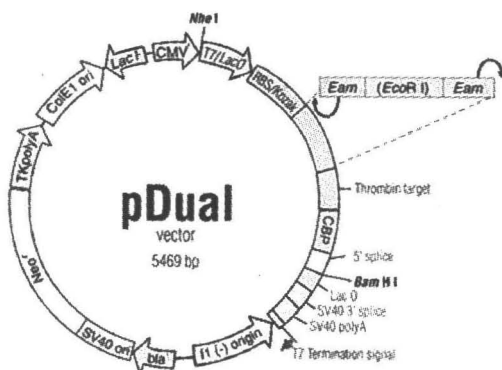
Such an evolutionary pathway would create a series of advance, each increasingly improbable, as indicated by the scale shown in table 3-for. As implied by the table, it is virtually certain that many cells in the transplant will express infectious PERV following transplantation and likely that some local infection of host cells might occur. The subsequent events necessary for generation of pathogenic, transmissible viruses are increasingly unlikely, what on some unknown, arbitrary scale. Although the probability of an inadvertent creation of a new epidemic is generally judged to be extremely small (particularly given the long history of intermittent association between humans and pigs), it cannot be ignored altogether. Current FDA policy is to permit xenotransplantation trials to proceed, but to require close monitoring of recipients and (insofar as possible) of their contacts. Attempts are also being made to identify specific proviruses responsible for production of infectious virus and then to selectively breed them out of lines of animals to be used as transplant donors.

## **In Single Vector Mammalian and Bacterial Expressions**

The vector contains the promoter and enhancer region of the human

cytomegalovirus (CMV) immediate early gene for constitutive expression of cloned DNA inserts in mammalian cells. Expression of heterologous genes in bacteria is regulated by a hybrid T7/ lacO promoter. The pDual vector was engineered to carry the lac repressor gene (lacI). Expression is therefore inducible using IPTG in bacteria that contain the T7 RNA polymerase. In both bacterial and mammalian cells, the dominant selectable marker is the neomycin phosphotransferase gene under the dual control of the -lactamase and SV40 promoters. A distinguishing feature of the pDual vector is the tandemly arranged bacterial Shine-Dalgarno1 and mammalian Kozak 2 consensus sequence. Each ribosome-binding site is positioned at its optimal distance from the initiation codon of the target gene, which ensures efficient translation of mRNA generated in either system. Mammalian expression vectors contain similar elements that may also be present in bacterial expression vectors. Yet mammalian and bacterial plasmid vectors vary considerably in their choice of promoters, the presence of splice signals and polyadenylation sites, as well as their respective translation initiation sequences. In fact, the differences between bacterial and mammalian expression vectors are so significant that, until now, these vectors could not be used interchangeably.

For expression in mammalian cells, eukaryotic genes are typically cloned first into a bacterial vector and then subcloned into a vector suitable for eukaryotic expression. Each step involves isolation and characterisation of clones containing the gene of interest, requiring a significant investment of time and biological reagents. Stratagene has now eliminated the need to subclone from one



vector system to another by combining the essential features of both in a dual expression vector.

The novel pDual vector can achieve high-level expression of heterologous genes in both mammalian and bacterial cells.

The pDual expression vector can be used in conjunction with the seamless cloning technique.<sup>3,4</sup> PCR amplification of the gene of interest, using primers that contain the *Eam*1104 I restriction sites and a minimal flanking sequence, permits rapid and efficient cloning in the presence of the type IIS *Eam*1104 I restriction enzyme. After digesting the PCR product with *Eam*1104 I, the fragment can be inserted via a three nucleotide 5' overhang that encodes the gene's own ATG. The use of the type IIS *Eam*1104 I restriction enzyme eliminates any primer-related residual nucleotides that are generally present when regular restriction enzyme recognition sites are encoded by the PCR primer sequences.

Taking advantage of the versatility of PCR cloning, the pDual vector offers two options for expressing a target gene. The reverse primer can be designed to contain a stop codon, which results in the target gene product being expressed in its native form. Alternatively, by designing a reverse primer that eliminates the gene's natural termination codon, researchers can fuse their target gene to the small calmodulin binding peptide (CBP) affinity tag located downstream of the cloning site. The 4-kDa CBP fusion tag, expressed on the C-terminus of the protein, can then be used for easy detection and purification of the fusion protein using Stratagene's AffinityCBP fusion detection kit and Calmodulin Affinity Resin, respectively. The new pDual expression vector is offered either in supercoiled form or predigested with *Eam*1104 I.

## **Prokaryotic and Eukaryotic Expression of the Green Fluorescent Protein**

We chose the green fluorescent protein<sup>5</sup> (GFP) gene as the reporter because its expression can be visualised easily in bacterial colonies, using a long-wave UV light source and in transfected tissue culture

cells, using standard fluorescence microscopy. Stratagene's Seamlesscloning kit<sup>4</sup> was used to amplify the GFP gene from the original TU585 construct using high-fidelity Pfu DNA polymerase with primers that contained the Eam1104 I recognition site. The digested vector and insert were ligated in the presence of the Eam1104 I restriction enzyme, eliminating the need for gel purification of the restriction fragments. Epicurian ColiXL1-Blue supercompetent bacteria were transformed with the ligation product and 18 colonies were screened by PCR for the presence of the reporter gene. One hundred percent of the screened colonies contained the desired insert and one of the clones, designated KPdual-1, was chosen for further studies. To test the ability of the pDual vector to express the GFP reporter in both prokaryotes and eukaryotes, we introduced the KPdual-1 construct into BL21(DE3) competent cells, which express the T7 RNA polymerase and into mammalian tissue culture cells, respectively. The pDual vector is able to direct expression of the reporter in both systems. The presence of the CBP-tagged GFP fusion protein in bacterial and mammalian cell lysates was further verified by Western blot analyses using GFP antiserum and the AffinityCBP fusion detection kit (data not shown).

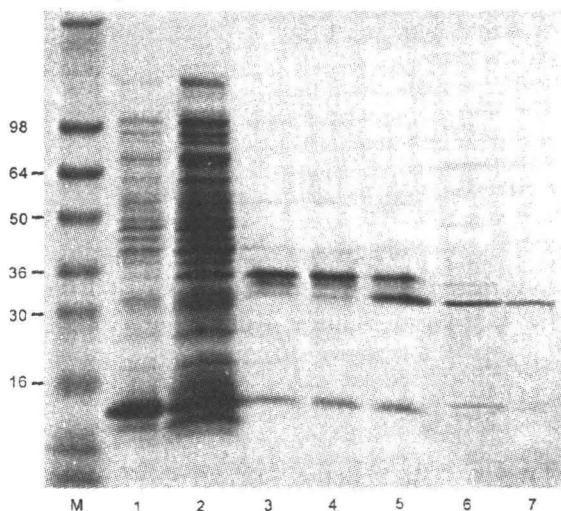
## **Inducible Expression of GFP-CBP Fusion Protein in BL21(DE3) Cells**

The hybrid T7/lacO phage promoter was tested by carrying out induction studies using IPTG. BL21(DE3) cells harbouring the KPdual-1 construct were grown in LB broth supplemented with 50 g/ml of kanamycin until the culture reached an OD<sub>600</sub> of approximately 0.6. One-half of the culture was then induced by adding IPTG to a final concentration of 0.2 mM. The induced and uninduced cultures were harvested after an additional 3-hour growth period at 37°C. Lysates were prepared by standard methods,<sup>6</sup> and the total protein concentration was determined by Bradford assay. Equal amounts of protein from IPTG induced and uninduced bacterial lysates were resolved by SDS-PAGE, transferred to nitrocellulose and visualised by chemiluminescence using GFP antiserum.

## Purification of CBP-Tagged Protein

The GFP-CBP fusion protein was purified from bacterial cell lysates using the Affinity purification system. The purification was performed using the batch method described in the manual supplied with the kit. The calmodulin affinity resin with adsorbed GFP-CBP fusion protein was applied to a disposable column, washed and then eluted at neutral pH as specified in the manual. Aliquots of the IPTG-induced and uninduced bacterial lysate and the eluted fraction containing the fusion protein were resolved by SDS-PAGE and visualised by staining with Coomassie brilliant blue dye.

A representative cleavage profile of the GFP-CBP fusion protein is shown in the figure above. The size of the GFP-



CBP hybrid protein is approximately 32 kDa, compared to that of the untagged 28-kDa GFP. The results indicate that the small CBP affinity tag, located on the C-terminus of the protein, provides an effective and convenient way to purify the protein of interest from bacteria. In addition, the thrombin protease cleavage site allows cleavage to occur in the presence of thrombin for applications where removal of the affinity tag is desired. A time-course experiment showed that successful cleavage was achieved when the purified GFP-CBP fusion protein was incubated at room temperature with as little as 2 ng of thrombin in the presence of calcium. Full-length fusion protein was present in an aliquot that was removed immediately after addition

of thrombin (lane 4), approximately 50% of the CBP fusion tag was cleaved after a 6-hour incubation at room temperature (lane 5) and approximately 95% of the fusion tag was cleaved after 24 hours (lane 6).

The pDual expression vector was developed to allow simultaneous expression of proteins in prokaryotic and mammalian systems. The vector contains a hybrid bacterial promoter for inducible bacterial expression, the CMV promoter and enhancer region for constitutive mammalian expression and tandem consensus sequences for optimal translation initiation in both systems. The cloning site is compatible with the novel Seamless cloning technique.<sup>3,4</sup> The CBP-affinity tag is located downstream of the cloning site, allowing simple detection and purification of a protein of interest. The CBP-affinity tag can be removed from the fusion protein via the thrombin cleavage site that immediately precedes the tag.

The pDual vector's unique cloning site consists of two Eam1104 I recognition sequences in opposite orientations, separated by a short spacer region. The CMV promoter drives expression in mammalian cells, whereas the hybrid T7/lacO promoter provides inducible expression in bacterial cells that express the RNA polymerase of bacteriophage T7. The Shine-Dalgarno and Kozak consensus sequences are positioned immediately adjacent to the Eam1104 I cloning site for efficient translation initiation in either bacteria or mammalian cells. The CBP fusion tag is located downstream of the Eam1104 I cloning site and is preceded by the target site for thrombin protease cleavage.

The Western blot analysis shows IPTG induction performed on BL21(DE3) cells transformed with the KPdual-1 construct. Equal amounts of total protein (6 g) were resolved and GFP was visualised by chemiluminescence using commercially available GFP antiserum and HRP-labelled secondary antibody. The biotinylated molecular weight protein markers were visualised by adding dilute streptavidin-labeled HRP to the secondary antibody solution. Lane M: Biotinylated molecular weight protein markers. Lane 1: IPTG induced bacterial cell lysates. Lane 2: Uninduced bacterial cell lysates. Lane 3: Commercially available recombinant GFP for size comparison.



## **Animal Medicines, Vaccines and Diagnostics**

Recent developments in the field of biotechnology have brought about a major change in the field of using vaccines and medicines. Biotechnology has yielded new and improved medicines for animals that help lower production costs and improve animal well being by fighting diseases caused by bacteria and parasites. For example, scientists have identified a new anti-bacterial compound that may serve as a substitute for using some antibiotics in animals, a practice that has been criticised for contributing to the increased prevalence of drug-resistant bacteria in human infections. Developed through modern biotechnology techniques, vaccines are now used to prevent diseases including: foot and mouth disease, scours, brucellosis, shipping fever, feline leukemia, rabies and infections affecting cultivated fish.

Biotechnology has led to the development of rapid test kits to diagnose the health of livestock and companion animals. Some kits are commercially available, but they will have to be low cost and easy to use if they are to be widely accepted.

## **Genes and the Genome**

Genes are found within the genome and serve as the “words” of the instruction manual. When a cell reads a word (expresses a gene), a specific protein is produced. Proteins give an individual cell and therefore the animal, its form and function. Genes (words) are written using the four-letter alphabet A, C, G, T. The letters stand for four chemicals called bases, which together compose DNA.

Animals are made of billions of cells all working together. Every cell of the animal has a complete “instruction manual” or genome (pronounced “JEE-nōm”) that is inherited from the parents of the animal as a combination of their genomes. The genome resides in the nucleus of the cell.

DNA is universal in nature, meaning that the four chemical bases of DNA are the same in all living organisms. Consequently, a gene from one organism can function in another organism. When a new gene is

put into an animal's genome it is said to be transgenic. Scientists have also discovered how to genetically modify (GM) when and/or where in an animal a gene is expressed. For example, it is possible to produce new proteins in the milk of a cow and nowhere else in its body.

## **Making a Transgenic Animal**

One way to produce transgenic animals is through a technique called microinjection. Once scientists have identified and isolated the piece of DNA comprising the gene to be transferred, it is injected into a fertilised egg of the desired animal using a very small glass needle visualised under a microscope. In approximately one percent of the injected eggs, the gene becomes a new “word” in the egg’s “instruction manual” by physically combining with the egg’s genome. Ideally, the new gene integrates into the genome before the egg begins to divide. If this occurs, every cell in the animal can contain the new protein and the animal will pass the gene on to its offspring. After injection of the gene, the fertilised egg is implanted into a surrogate mother where it fully develops into a transgenic animal.

Currently, the only routine commercial use of transgenic animals (primarily mice) is in the area of human disease research. One way to characterise the range of genetic modifications that are being considered for use in animals is in the three broad areas of input, output and value-added traits. Examples of each are described below.

## **Input Traits**

An “input” trait helps livestock and dairy producers by increasing production efficiency.

Input traits that are being investigated for use in animals:

- Faster, more efficient growth rates
- Increased production of milk or wool
- Resistance to diseases caused by viruses and bacteria

## Output Traits

An output trait helps consumers or downstream processors by enhancing the quality of the animal product.

Output traits that may prove to be beneficial:

- Leaner, more tender beef and pork
- Milk that lacks allergenic proteins or results in increased amounts of cheese and yogurt

By adding or modifying genes, animals can function in completely new ways.

- Producing large amounts of therapeutic proteins in animal milk may be an efficient, relatively low cost method to manufacture many proteins used to treat human diseases or proteins that have industrial value.
- Transplanting animal organs into humans or xenotransplantation, can be made more successful by genetically modifying the organs so that they are not as readily rejected by the human immune system.
- Development of animals that serve, as models for human diseases to help scientists better understand prevention and treatment strategies.

Proteins with therapeutic and industrial value that have been produced (but not commercialised) in the milk of transgenic animals include:

Proteins	Animals	Use
Antithrombin III	Goat	Reduce the amount of blood needed in some surgeries
Factor VIII, Factor IX	Goat, Pig, Sheep	Treatment of hemophilia
CFTR	Sheep	Treatment of cystic fibrosis

Lactoferrin	Cow	Natural antibiotic and used in coronary surgery
Alpha-1-antitrypsin	Sheep	Treatment of cystic fibrosis and emphysema
Lysostaphin	Cow	An anti-bacterial compound that prevents mastitis in cows
Spider silk protein	Goat	Production of ultra-strong, lightweight medical and industrial materials

## Risks and Regulation of Transgenic Animals

One concern of transgenic animal technology is the welfare of the animals. Developmental and health abnormalities have been reported in conjunction with its use; therefore, researchers must take care to minimise animal suffering.

The inadvertent release or escape of transgenic animals (particularly fish) into the wild where they could breed or compete with the natural population is often cited as a potential risk to the environment. The actual risk associated with this will depend on the type of animal and the nature of the genetic modification; however, where appropriate, procedures must be in place to alleviate this concern. At the Federal level, the Food and Drug Administration, the Department of Agriculture and the Environmental Protection Agency are required to regulate transgenic animals and their products to ensure that they are safe for public use and the environment. Depending on the nature of the genetic modification and the proposed use of the resulting animal or product, more than one agency may be involved in the approval process.

## Transgenic fish and shellfish

Aquaculture, worth an estimated \$46 billion in 1997, produces

approximately one-third of all fish and shellfish consumed by humans. As of January 2002, the FDA was considering petitions to allow the commercial use of transgenic salmon and trout with enhanced growth characteristics. In addition, researchers are working with transgenic catfish, carp, tilapia, striped bass, clams, oysters, shrimp and abalone.

Traits being tested in transgenic fish include:

- Growth rates that are 3-11 times faster with more efficient feed utilisation
- Increased tolerance to cold water
- Improved disease resistance

Safety and risk issues associated with transgenic fish:

**Issue:** Introduction of a protein that is potentially allergenic or toxic to humans

**Solution:** Extensively test before the FDA grants market approval  
**Issue:** Escape of a transgenic fish into the wild, leading to interbreeding and/or competition with the natural population.

**Solution:** Restrict cultivation to land-locked facilities or guarantee sterility of transgenic species

## Biotechnology and Animal Cloning

Advances in biotechnology have allowed scientists to make genetically identical copies or clones of animals. Duplication of an organism's genome occurs naturally when identical twins are born or when a plant is grown from a cutting of another plant. However, the world really took notice of cloning in 1997 when a group of Scottish researchers announced the birth of Dolly the sheep, which had been cloned using a single cell from an adult sheep. Dolly had only one "parent;" her nuclear genome was exactly like her "mother's" instead of being a combination of two parents. Therefore, Dolly could generally be thought of as her mother's identical twin.

To produce Dolly, scientists took an egg from a sheep and removed its nucleus (which contains the genome or instruction manual),

rendering it unable to function or develop. Next, they took a cell with an intact genome from a different adult sheep (Dolly's "mother") and fused it to the sheep egg, which lacked a genome. The egg, with its new genome, was stimulated to begin developing into an embryo and was implanted into a surrogate sheep where it grew normally, resulting in the birth of Dolly. Dolly later gave birth to normal lambs.

## Benefits and Risks of Cloning

Researchers have cloned other mammals including cows, goats, pigs and mice. However, the overall low rate of successful cloning and frequent occurrence of developmental abnormalities in cloned animals demonstrate the need for further research before cloning will be practical. It has also been reported that cloned animals may exhibit health problems throughout their life. Cloned animals may age prematurely as Dolly was diagnosed with arthritis at a seemingly young age and cloned mice had a shorter than normal life span. Additionally, it was demonstrated that cloned mice were both larger in size and heavier than a control group of non-cloned mice. If advances in animal cloning technology were to overcome the current obstacles, the most obvious benefit would be the ability of a farmer to have a herd of superior performing animals in one generation. Breeding companies could sell cloned embryos in a manner similar to the way in which semen is currently marketed. A potential drawback of this practice would be the loss of genetic diversity in livestock herds, but this could be avoided by limiting the number of cloned embryos of a given animal that were sold. It has also been proposed that cloning could be used to increase the population of animals in an endangered species. The mouflon sheep, which is a wild Mediterranean sheep with less than 1000 animals remaining, was successfully cloned. Additionally, scientists are attempting to clone an endangered wild Asian ox, called the guar (the first cloned guar died of an intestinal illness shortly after birth) and possibly the giant Panda. Although possible, a recovering population of cloned animals would be hindered by a lack of genetic diversity and would not address the larger issue of how the animal became endangered.

## **Benefits of Animal research**

Now a days we no longer have to fear the many common diseases which were the main causes of childhood death only a century ago. These diseases have been conquered by medical research. Despite the enormous medical advances of the last 100 years, there are still many serious illnesses for which no effective treatment is known. Continuing medical research is the hope for future generations. A great many medical breakthroughs have depended on the use of laboratory animals and much of the medical research being done today still depends on them. Yet this research faces increasingly hostile campaigns by those who, through lack of understanding, would seek to ban all animal research. It is very important that we examine the facts. For every £1 spent on medical research, only 5p is spent on animal experiments, which are vital and complement computer studies, test tube experiments and studying people. If we look at the number of laboratory animals used and the potential improvements in quality of life for both humans and other animals arising from such research, then we can see that the benefits far outweigh the costs. The following facts bring animal experimentation into a realistic focus:

- It is estimated that two million cats and dogs are abandoned as unwanted pets every year and even more animals are destroyed as vermin. The RSPCA alone has to handle 2,000 cats and dogs every day
- Last year 800 million animals were slaughtered for human consumption but only 2.6 million animals were used in medical research. That's roughly equivalent to one mouse per person every 20 years
- Of all the areas of animal use, including agriculture, pets, sports and animal research, the standards of welfare and veterinary care laid down are the highest for animals kept in laboratories for research.

## **Vaccines and Viral Infections**

Human viral diseases include the common cold, influenza and more

recently AIDS. Other viral diseases like smallpox, polio, mumps and measles are preventable due to vaccines developed with animal experiments. Viruses can invade many parts of the body, causing serious harm to vital organs such as kidney, liver or brain. The emergence of AIDS illustrates the constant and urgent need for ways of combating new viral infections. In Britain about 20,000 people are diagnosed as HIV positive. Several animal species are infected by similar immune-efficiency viruses and the study of these is vital to the understanding of HIV and AIDS that will lead to effective treatments or vaccines. Already there are 15 anti HIV drugs and a vaccine is now in clinical trials. The current aim is to stop the virus multiplying and hopefully disarm it without harming the host cells. Since 1978, not a single person in the world has caught smallpox. That's because almost everyone was vaccinated against it. Now that smallpox has disappeared children today don't need to be vaccinated against it. The vaccine had to be obtained from calves and was tested on animals to make sure it was safe. Before children were given polio vaccinations about 30,000 children in North America and Europe caught polio every year.

There are about a million people alive today who would have caught polio if they had not been vaccinated. The vaccine was developed using animal in the 1950s and then finally tested on monkeys, to make sure it was effective and safe. Vaccines for immunisation against the six most common childhood diseases - measles, diphtheria, whooping cough, tetanus, polio and tuberculosis - are saving the lives of over 3 million children every year or six every minute, says UNICEF. In 1990 more than 100 million children under the age of one year were successfully immunised and the UN Secretary General announced that eight out of every ten of the world's children are now immunised. That still leaves two million children who die each year because they are not immunised.

## **Antibiotics and Bacterial Infections**

Antibiotics have revolutionised the treatment of life threatening conditions and now they can be treated with a good rate of recovery. The introduction of the first antibiotic, penicillin, in 1941, saved



millions of lives. Animals have played an important role in our understanding of bacterial disease and in developing antibiotics. Nowadays, potential new antibiotics are investigated initially in test-tubes. But they must be evaluated for safety and effectiveness in animals before being tested and used in people.

## **The Dangers of Cancer**

In Britain, 440 people die from cancer every day, three of these will be children under 15. However, two main advances have increased chances of survival. One of the first breakthroughs was the development of radiotherapy, in which radiation kills the tumour without causing too much damage to the rest of the body. Then came chemotherapy - drugs that kill the rapidly dividing cancer cells. Both of these techniques were developed and tested with the help of animal experiments. For the 700 or so people in the UK this year who will develop a type of cancer called Hodgkin's disease, the advances in treatment mean that 500 of them will still be alive in five years. Without any treatment people with Hodgkin's disease always die. Similar progress has occurred in other cancer studies. Today 6 out of every 10 children with leukaemia can be cured. Overall four out of ten people who develop cancer are now cured. Twenty years ago, the figure was two out of ten.

## **Heart Disease**

Coronary heart disease is a major cause of suffering and premature death. It kills one person in four in Western societies despite significant advances in treatment over the last 40 years or so. The development of the heart lung machine in the 1940s made open heart surgery possible. About a third of a million pacemakers are implanted annually worldwide to regulate faltering heartbeats and in this country about 6,000 operations are carried out every year to repair or replace faulty heart valves. In the 1960s a major new surgical technique was developed to by-pass damaged arteries and some 22,000 coronary by-pass operations are now performed in Britain every year. More recently,

heart transplants have enabled some patients to enjoy many more years of active life: about 350 are carried out every year, with a 90% success rate. This is helped by immunosuppressive drugs which are used to prevent rejection after all organ transplant operations. Beta-blocking drugs to reduce high blood pressure and prevent heart attacks were developed in the late 1950s.

Animal research was essential in the development of all these treatments. Thanks to vaccines and antibiotics, infectious diseases are no longer life threatening, in the UK at least. But in the third world, parasitic disease is still a major killer. Malaria has increased dramatically in recent years. Nearly 300 million people are infected with malaria and two million die every year. Treatment and control are difficult because of drug-resistant strains of malaria and insecticide-resistant strains of mosquito. Finding a vaccine is the top priority but potential vaccines must be safe.

Animal tests are the vital link between the test-tube and people in this research.

## **Kidney Disease**

Over 3,000 people a year in the UK develop kidney failure. A third of these would die without regular dialysis on a kidney machine or a kidney transplant.

The kidney machine, which removes toxic waste products from the blood, was a direct result of work on rabbits and dogs. It is vital during dialysis to prevent the blood clotting as it passes through the machine and this is achieved by adding the drug heparin. The anti-clotting action of heparin was discovered by experiments in dogs and today the drug is obtained from beef liver. Transplant surgery often offers the only real hope for kidney patients and over 33,500 successful kidney transplants have been carried out in the UK since the technique was first developed using dogs in the 1950s.

Again, anti-rejection drugs, developed by testing their ability to prevent rejection of grafts in mice, are vital to the survival of the transplanted

organ. Now, nearly 2,000 kidney transplants are performed every year in Britain.

## **Asthma**

It affects one in seven schoolchildren though many cases are not being properly diagnosed. Severe asthma causes tremendous suffering and can be lethal; last year it killed 2,000. There are drugs which relieve the symptoms of asthma, but do little to prevent the underlying worsening of the disease. New drugs will require very thorough development, refinement and research on animals for both effectiveness and safety.

## **Alzheimer's disease**

Alzheimer's disease is a progressive brain disorder affecting about 700,000 people in Britain. It is very disturbing to see the elderly and sometimes not so elderly, suffering from almost complete memory loss, a symptom of this illness. Research on mice has already demonstrated that abnormalities (structures called plaques) seen in the brains of Alzheimer's patients are due to the formation of a particular protein. This mouse model of Alzheimer's disease, produced by genetic engineering, will prove invaluable in developing ways of treating or preventing the disease.

## **Epilepsy**

This unpredictable condition afflicts about 340,000 men, women and children in Britain: that's about 1 in every 200 people. Today's medicines offer several effective treatments. Usually just one medicine is sufficient, but sometimes a combination has to be used. Even so, in one in five cases, no treatment seems to work. Research into understanding the function of the brain and the changes which occur during epileptic conditions must go on, if new anti-epileptic medicines are to be discovered. Much of what is known today about epilepsy has been gained by studying animals, particularly the mouse.

## Veterinary Disease

Dogs in particular suffer from a variety of diseases: distemper, infectious hepatitis, leptospirosis, kennel cough and parvovirus infection can be controlled by vaccines, which were all developed with animal experiments. A typical example of the efficiency of such research is that for each laboratory animal used in the research, 75,000 dogs can now be protected for life against distemper. Cats suffer from cat flu, feline enteritis and leukaemia, all of which can be prevented by vaccinations. Many of the medicines used to treat animals - antibiotics, painkillers, anaesthetics, tranquilisers - are exactly the same as those used to treat people.

## Genetic Diseases

The recent unravelling of the human genome will open the door to finding treatments for a wide range of disorders, not just the obviously inherited ones but also those with a genetic background, e.g. diabetes, cardiovascular disease, common cancers, the major mental illnesses and many more. The 5,000 diseases caused by a single gene defect will probably be tackled first. Since these affect one in every hundred infants born, that alone is a massive task. Work on mouse models of diseases continues to make major contributions to knowledge of human genetics. The genetic constitution of mouse and man are surprisingly similar. Genetic modification techniques (transgenesis) allows a disease-causing human gene mutation to be inserted into a mouse's genome. Transgenic mouse models enable researchers to study the complex interactions, at every stage of life, within a whole living environment. It is remarkable that, during the last 20 years, the genes responsible for nearly all the relatively common inherited diseases have been located and isolated. For example, Duchenne muscular dystrophy, cystic fibrosis, haemophilia, neurofibromatosis, Huntingdon's disease, infantile spinal muscular atrophy and many others.

Genetic research, using mostly the mouse, is set to revolutionise our

medical understanding and gives hope of finding cures where previous hope did not exist.

## **Muscular Dystrophy**

Two male children are born with Duchenne muscular dystrophy every week in Britain. Their muscle cells lack an essential substance so they gradually develop muscular weakness in infancy and will probably die in their mid 20s. The exact genetic abnormality found in sufferers is also found in some mice and research using these mice is providing valuable clues to the condition and to potential treatments.

## **Occurrence of Abnormalities**

Severe anaemia occurs when the red blood cells cannot deliver enough oxygen to different parts of the body. They are the most common inherited disorders caused by a single gene defect. It is estimated there are over 6,000 people with the two important haemoglobin disorders, sickle cell anaemia and thalassaemia. The recent development, by genetic engineering, of a mouse model of sickle cell disease should enable doctors to understand why the red blood cells become deformed and to develop new approaches to treatment.

## **Autoimmune Disorders**

Several serious disorders are the result, either wholly or partially, of the defences of the body turning on themselves. These disorders have proved particularly difficult to understand and treat. With the help of animal models, doctors are now making progress on many fronts.

## **Rheumatism**

This is the biggest single cause of disablement and it can affect the whole of people's lives: even maintaining independence can be an anxious and continual battle. There are as many as 200 different kinds of rheumatic disease: the better known ones being rheumatoid arthritis (with a million sufferers in the UK), osteoarthritis (with 5-10 million

sufferers), gout and ankylosing spondylitis. Great advances have been made, although there is as yet no cure for rheumatic disease. Research is aimed at different aspects of the problem; understanding the causes and mechanisms of the disease, new surgical techniques, new materials for replacing affected joints (there are about 50,000 hip replacements every year in the UK) and new medicines to control and alleviate symptoms. Rats, rabbits and guinea pigs have been used to increase understanding of rheumatic disease.

## **Multiple Sclerosis (MS)**

This is a disease of the central nervous system that strips the protective myelin insulation from nerves within the body. This causes deterioration of body functions, such as muscle movement, balance, strength, speech and vision. It is estimated that about 80,000 people have MS in Britain. Researchers have learned a lot about MS through a disease called experimental autoimmune encephalomyelitis. This laboratory induced disease, very similar to MS, occurs in rats, mice and guinea pigs. Using these animal models, researchers have studied the basic biological problems related to MS and have shown that it may be possible to 'switch off' the disease by treatment with antibodies and to transplant myelin-making cells, which can repair damaged nerves. These models have also been used to test whether potential MS drugs are effective and safe.

## **Cause of Diabetes**

Diabetes is thought to be caused by a number of different factors - environmental, genetic and autoimmune. Eighty years ago, to be diagnosed diabetic was a death sentence. If you were young, you probably had less than a year to live. It is estimated that over 10 million diabetes sufferers have been saved from death since insulin was introduced. Today, in Britain alone, there are 1,400,000 people with diagnosed diabetes. Another 600,000 people don't yet know they're diabetic. In diabetes, the pancreas cannot produce its own insulin, the hormone that enables the body to use sugar. Insulin was

first isolated from the pancreas of dogs by Frederick Banting and Charles Best at the University of Toronto in 1921. Insulin is a protein found in all animals and, up until recently, diabetics used insulin produced from the cow and pig pancreas. It is purified and then standardised by testing in mice.

For each animal used, enough insulin is produced to treat 70 diabetics for a year. Insulin saves lives, every day, by controlling diabetes and enabling people to live more or less normally. But it is a treatment, not a cure. Research continues, using mouse and rat models, with the aim of finding safe and effective treatments which do not require daily injections. Ultimately, the aim of the research is to find a prevention or cure: current studies indicate that transplants of insulin-producing cells from animals may be possible in the future.

## Gene Therapy

If faulty genes, i.e. DNA that doesn't code for the correct amino acids, cause a disease then why not replace the faulty genes with copies that work. First of all, we need a copy of the gene. This is the essential first step but has been difficult. The reason is that there are around 100,000 different genes in humans. For many diseases, the genes responsible are unknown. Thankfully, this is changing - and changing fast. The Human Genome Project is an international project, involving scientists in many countries. The object is to produce a complete map of all of the genes that make up humans, including the sequence of 'letters' for each gene. This has led to many more genes being identified and associated with different diseases. Once we know what the gene is, it needs to be cloned. 'Cloning' is one of those words that seems to crop up everywhere these days and in different contexts. Many people are confused when they hear of DNA being cloned and then also of animals being cloned. How can such different things all be clones? The reason is that 'clone' simply means 'copy' and to clone something just means to make exact copies of it. Gardeners do this all the time when they take cuttings from a plant and grow them on. In the case of genes, cloning means that a copy of the gene is taken from a sample of human

or animal DNA and inserted into yeast, bacteria or virus. This allows many copies of the gene to be easily made and used in more complicated ways. What we are talking about here is getting enough copies of a “good gene” so that it can be used to treat patients who are suffering from diseases caused by a “bad” version of the same gene. Once we have enough copies of the gene, we need to get it into the right cells in the patient and make sure it produces the required “good” protein in sufficient quantities. This has turned out to be the most difficult step of all. The gene has to be manipulated: it needs various other bits of DNA attached to it to make sure that this happens and it needs some sort of vehicle to carry it into the target cells in the body. It should be stressed that all gene therapy being developed at the moment is somatic gene therapy. This means that it is designed to change only the body’s ordinary cells. Germ-line therapy - changing the DNA of eggs, sperm or embryo so that offspring will also contain the new DNA - is not being developed because not enough is known about possible adverse effects in future generations.

Two main approaches for getting DNA into humans have been tried - viruses and liposomes. The idea of using viruses to cure diseases may sound strange - after all, they cause them! Well, think about what viruses are and what they do; they are small particles, which contain nucleic acid and gain entry to the cells in the body by infecting them. This makes viruses good candidates for use in gene therapy - since that is exactly what we want to do; get DNA into cells. The idea is that if the viral DNA can be replaced with the useful human DNA, then the virus could infect the body with the good gene, rather than a disease causing gene.

In fact, it has been suggested that a modified version of the AIDS virus, HIV, would be useful for this. However, viruses are not the only potential vehicle for gene therapy; scientists are also looking at the use of liposomes. These are essentially tiny globules of fat - the same sort of fat as surrounds cells - with DNA inside. The liposomes fuse with cells and release their DNA into them. These are thought to be a potentially gentler way of introducing genes to the lungs for instance, than using viruses.



## Gene Pharming

Aside from developing models for human genetic diseases, there are two other main reasons why changing the DNA of animals could be useful - and both are at an even more advanced stage than gene therapy.

The first one is making animals produce medically useful compounds; 'pharming'. This is done by cloning the human gene into animals. Why not just use tissue cultures to get the product we want? One reason is that cells don't produce much of the products of most genes. Since there are many different types of cells, each only makes products from a proportion of genes. Genes come with additional information that tells the cell when it should be used to make protein and how much should be made. In tissue culture there is only one type of cell and it is not always possible to persuade them to produce large amounts of the desired protein. It is also costly - an important factor when considering producing treatments for rare diseases. One reason that cell culture is so costly is that it has a requirement for highly specified animal-derived ingredients such as foetal calf serum.

So how could we use biotechnology to get around these problems? The answer is brilliantly simple. Animals make a lot of the types of protein that are produced in milk. So one approach is to clone the desired gene into an animal, along with additional genetic information that says 'produce a lot of this protein in milk'. This is a good way of producing large amounts of the desired protein, since instead of an expensive tissue culture process, dependent on animal culture, we have a living animal. Grass gets fed in and milk is produced containing valuable proteins in large amounts.

The difficulty is in producing the genetically engineered animals in the first place. This is done by inserting the human gene into embryonic cells and are implanted into the wombs of mother animals where they grow and are born in the normal way. Then a second problem arises. If we have only one sheep, say, producing this valuable milk then how can we ensure continued supply or increase the supply, of the milk?

This is where cloning comes into the picture again. Only instead of

producing copies of a gene, we can produce copies of whole animals. This is what has been done with Dolly, the famous cloned sheep. Such cloning involves taking a single cell from the 'parent' animal. Scientists discovered a way in which this single cell could be persuaded to develop and grow into a new animal - just like a fertilised egg does. The difference is that the new animal is an exact copy of one 'parent'. So if the 'parent' produces a valuable pharmaceutical compound in its milk, so does the clone. Cloning is a good way of making the genetically modified founder animals for a flock producing therapeutic compounds.

Already 'pharming' is being used to produce some potentially valuable treatments for conditions like cystic fibrosis, haemophilia, emphysema and Pompe's disease. Yet there is fierce opposition from anti-vivisectionists who have concentrated on the negative aspects of the cloning technology such as surgical interventions and low survival rates. They certainly cannot attack it on animal husbandry grounds - such animals are just like ordinary farm animals - except that, as they are much more valuable, they are arguably much better looked after.

## **The Basics of Monoclonal Antibody Technology**

Substances foreign to the body, such as disease-causing bacteria and viruses and other infectious agents, known as antigens, are recognised by the body's immune system as invaders. Our natural defences against these infectious agents are antibodies, proteins that seek out the antigens and help destroy them. Antibodies have two very useful characteristics. First, they are extremely specific; that is, each antibody binds to and attacks one particular antigen. Second, some antibodies, once activated by the occurrence of a disease, continue to confer resistance against that disease; classic examples are the antibodies to the childhood diseases chickenpox and measles. The second characteristic of antibodies makes it possible to develop vaccines. A vaccine is a preparation of killed or weakened bacteria or viruses that, when introduced into the body, stimulates the production of antibodies against the antigens it contains.

It is the first trait of antibodies, their specificity, that makes monoclonal

antibody technology so valuable. Not only can antibodies be used therapeutically, to protect against disease; they can also help to diagnose a wide variety of illnesses and can detect the presence of drugs, viral and bacterial products and other unusual or abnormal substances in the blood. Given such a diversity of uses for these disease-fighting substances, their production in pure quantities has long been the focus of scientific investigation. The conventional method was to inject a laboratory animal with an antigen and then, after antibodies had been formed, collect those antibodies from the blood serum (antibody-containing blood serum is called antiserum). There are two problems with this method: It yields antiserum that contains undesired substances and it provides a very small amount of usable antibody. Monoclonal antibody technology allows us to produce large amounts of pure antibodies in the following way: We can obtain cells that produce antibodies naturally; we also have available a class of cells that can grow continually in cell culture. If we form a hybrid that combines the characteristic of "immortality" with the ability to produce the desired substance, we would have, in effect, a factory to produce antibodies that worked around the clock.

In monoclonal antibody technology, tumour cells that can replicate endlessly are fused with mammalian cells that produce an antibody. The result of this cell fusion is a "hybridoma," which will continually produce antibodies. These antibodies are called monoclonal because they come from only one type of cell, the hybridoma cell; antibodies produced by conventional methods, on the other hand, are derived from preparations containing many kinds of cells and hence are called polyclonal. An example of how monoclonal antibodies are derived is described below.

A myeloma is a tumour of the bone marrow that can be adapted to grow permanently in cell culture. When myeloma cells were fused with antibody-producing mammalian spleen cells, it was found that the resulting hybrid cells or hybridomas, produced large amounts of monoclonal antibody. This product of cell fusion combined the desired qualities of the two different types of cells: the ability to grow continually and the ability to produce large amounts of pure antibody.

Because selected hybrid cells produce only one specific antibody, they are more pure than the polyclonal antibodies produced by conventional techniques. They are potentially more effective than conventional drugs in fighting disease, since drugs attack not only the foreign substance but the body's own cells as well, sometimes producing undesirable side effects such as nausea and allergic reactions. Monoclonal antibodies attack the target molecule and only the target molecule, with no or greatly diminished side effects.

## Introduction Of Novel Genes

A number of methods are presently employed for genetic engineering of various animal species. Most of these were developed originally in mouse and *Drosophila* models and have only more recently been extended to other domesticated animals. Access to the germline of mammals can be obtained by: (1) direct manipulation of the fertilised egg, followed by its implantation into the uterus; (2) manipulation of the sperm used to generate the zygote; (3) manipulation of early embryonic tissue in place; (4) the use of embryonic stem (ES) cell lines which, after manipulation and selection *ex vivo*, can then be introduced into early embryos, some of whose germline will develop from the ES cells and (5) manipulation of cultured somatic cells, whose nuclei then can be transferred into enucleated oocytes and thereby provide the genetic information required to produce a whole animal. The last two methods have the advantage of allowing cells containing the modification of interest to be selected prior to undertaking the expensive and lengthy process of generating animals. Usable ES cells are not available for all species of interest, however and generation of embryos by nuclear transfer (NT) from somatic cells is becoming the method of choice for genetic engineering and duplication of nearly genetically identical animals.

Manipulation of the avian germline is difficult since ES lines are not available and the early embryo is difficult to access. Much current work focuses on the use of blastodermal cells or primordial germ cells, which can be cultured briefly and manipulated to modify the

germline prior to introduction into fresh embryos to create chimeras from which modified lines can eventually be developed, albeit with low efficiency.

There are two basic approaches presently in use for inserting DNA into vertebrate germline cells, transfection and infection with retrovirus vectors. A third approach, based on the use of mobile genetic elements, has been commonly used for insects and is being explored for germline modification of vertebrates.

## **The Methods of Transfection**

Transfection methods include: (1) direct microinjection of DNA into the cell nucleus; (2) electroporation—introduction of DNA through transient pores created by controlled electrical pulses; (3) use of polycations to neutralise charges on DNA and the cell surface that prevent efficient uptake of DNA; (4) lipofection or enclosure of DNA in lipid vesicles that enter a cell by membrane fusion much in the manner of a virus and (5) sperm-mediated transfection, possibly in conjunction with intracytoplasmic sperm injection (ICSI) or electroporation. The manner of introduction of DNA is a technical issue, determined empirically for each system and makes little difference to the final outcome. In general the structure of DNA introduced into a cell by any of these methods is highly variable and uncertain. Often, only a fragment of the transfected DNA is integrated into the chromosome, frequently in multiple copies, that often are integrated in long tandem arrays. When transfecting cultured somatic or ES cells, a selectable marker, such as the gene encoding phosphotransferase, is often included as part of the DNA to permit selection for its presence either in eukaryotic cell lines or in the bacteria in which the DNA was mass-produced.

## **Retrovirus Vectors**

Retroviruses are infectious elements that replicate by a unique process involving copying of the viral RNA genome into DNA (a process

called reverse transcription) followed by its specific and stable introduction into host cell DNA (integration). The integrated DNA then can be expressed using the normal transcriptional machinery of the cell. Retroviruses commonly are used to introduce genes of interest into cells in culture or into somatic tissue in experimental animals. They also have been used for germline modification of fish mollusks chickens mice and, more recently, cattle .To make a retrovirus vector, a DNA construct containing the gene of interest is flanked by sequences necessary for replication as a virus. These sequences include transcriptional promoters in the long terminal repeats (LTR's), which flank the integrated DNA or provirus. Signals necessary for packaging of the transcript in virions (virus particles), for reverse transcription and for integration of the resulting DNA also must be included. Introduction of such a DNA construct into cells that express viral proteins, but that are incapable of making infectious virus (i.e., helper or packaging, cells), leads to the creation of infectious virions containing an RNA copy of the gene of interest. After infection of cells with such virions, the RNA is copied into DNA and integrated at random sites in the cell genome. Again, selectable markers often are included in the construct to select cells containing the desired virus construct.

## Transposons

Transposons are DNA elements that (in the presence of the appropriate gene products or transposases can transfer their information from one site to another in the same cell. A variety of transposons have been found in insects and some are routinely used as vectors for the generation of transgenic insects. No active transposons of these types have been observed in mammals, although the human genome contains thousands of copies of a DNA sequence related to the mariner transposon of *Drosophila* suggesting that there might have been active elements in our recent evolutionary history. Nevertheless, several recent reports suggest that naturally-occurring transposons found in insects or even bacteria, might provide a useful and efficient means of introducing genes into the germline of animals. Mariner, for example,

has been shown to be active in chick zygotes, transferring its DNA from a microinjected plasmid into the germline, albeit at low efficiency. A modified version of Sleeping Beauty, a related element from fish, has been developed to give a high yield of germline or somatic transformants in cultured cells and laboratory mice. In practice, it remains to be seen whether—and how efficiently—genes of interest can be transferred in this way. Another transposon system being investigated for this purpose is the T DNA of *Agrobacterium tumefaciens*, a natural pathogen of plants. This bacterium can fuse with plant cells, leading to transposition of the DNA (along with whatever genes it carries) into the nuclear DNA of the host. This technique is widely used for the generation of transgenic plants. Remarkably, it recently has been shown that *Agrobacterium* can fuse with human cells in culture, leading to transfer of T DNA carrying a marker gene. None of the transposon-based techniques currently are used for the generation of transgenic livestock, but they might lead to more efficient methods for this purpose.

## The Genetic Manipulation

Transgenic technology is the creation of engineered animals that lack specific genes (knockout) or have these genes replaced by one that has been engineered in a specific way. For example, transplantation of organs or tissues from non-primates (such as pigs) to humans (xenotransplantation) is currently impossible, due in part to a dramatic (“hyperacute”) immune response by human recipients to a carbohydrate on the surface of pig cells (galactose-1,3-galactose); this carbohydrate is not found in old-world primates. Inactivation of the enzyme (galactosyl transferase, GT) in donor pigs could alleviate this problem and pigs with one allele of the gene encoding this enzyme recently have been produced giving rise to expectation that completely GT-deficient animals soon will be available. Another important goal is to eliminate from cattle the gene encoding prion-related protein (PrP), the protein associated with scrapie in sheep and bovine spongiform encephalopathy. Removal of this gene from mice has, at most, subtle phenotypic consequences, yet renders them completely

resistant to these diseases. If the mouse model holds true in cattle, homozygous knockout of bovine PrP could lead to the elimination of RSE.

## **Knockout and Knockin Technology**

When a gene is altered but not shut down, a “targeted mutation” effect is created. This practice is referred to as knockin technology

In order to study the relationship between proteins and gene function, scientists now can prevent the manufacturing of a protein by a specific gene. By disabling a gene from a test organism and then producing descendants that contain two copies of the disabled gene, it is possible to observe the descendants’ development in the absence of a particular protein. This practice, referred to as knockout technology, is an attempt to shut down or turn off a particular gene. Thus far, the mouse has been the mammal in which knockout technology has been most generally applied. In essence, a “knockout” organism (e.g., the mouse) is created when an embryo cell (an embryonic stem cell—or ESC—which is a cell that has yet to divide into different tissue cells; is genetically engineered and then inserted into a developing embryo. The embryo then is inserted surgically into the womb of a host (e.g., a female mouse). Once the embryo has matured, a portion of its stem cells will produce egg and sperm with the knocked-out gene.

Gene knockout/knockin technology is well established as an experimental tool in mice due to the availability of ES cell lines. The principle is to take advantage of a rather rare event that occurs after introduction of DNA into cells—homologous recombination between identical sequences in the genome and the transfecting DNA. In the most common protocol, a selectable marker (such as the neomycin resistance gene) is inserted within a piece of DNA corresponding to a portion of a gene of interest. After transfection of cells by this construct and selection for the marker (by growth in a medium containing the neomycin-related antibiotic G418, in this example), the selected cells are screened to identify the small fraction that has one copy of the gene of interest disrupted by the marker. Progeny animals derived



from the cells will be heterozygous for the “knocked-out” or “knocked-in” gene; breeding to obtain homozygous animals is straightforward. Because the process is so inefficient, very large numbers of transfected cells must be screened, making the use of cultured cells essential, since it would be impracticable to screen large numbers of progeny from microinjected eggs. The galactosyl transferase-knockout pigs discussed above were generated from cultured foetal fibroblasts manipulated in this way. Nuclei from these cells then were transferred into oocytes as described in the next section.

### Propagation By Nuclear Transfer

In February of 1997, Dolly the sheep was introduced and the public subsequently was inundated with opinions about the power and potential of creating new animals from somatic cells. Dolly represented the most recent advance in genetic technology—the production of multiple individuals nearly genetically identical to an adult animal. In this technique, somatic cells from an appropriate tissue are grown in culture and their nuclei are injected into enucleated oocytes obtained from another individual of the same or a closely related species. After a further period of culture, the partially developed embryos are transferred into a foster mother. This technology is being developed rapidly for many species of interest and promises to become a rapid and efficient means of propagating domestic animals with desired traits, whether those are naturally derived and selected or genetically engineered. The process often is referred to as “cloning”. The nuclear transfer technique was based on previous studies in frogs conducted during the previous 5 decades., but, until Dolly, it was unclear whether nuclei from highly differentiated somatic cells could be reprogrammed to a pattern of gene expression suitable for directing normal development of a mammalian embryo.

TABLE

State of the art of transgenic technology for selected organisms

Organism	Transfection	Viral	Transposon	ES	Nuclear
			vectors	cells	transfer

Mouse	4a	2	1	4a	2
Cow	3	1	0	0	2
Sheep	3	0	0	0	2
Goat	3	0	0	0	2
Pig	3	0	0	0	2
Rabbit	3	0	0	1	0
Chicken	1	2	1	0	0
Atlantic salmon	3	0	0	0	
Channel catfish	2	0	0	0	
Tilapia	3	0	0	0	0
Zebrafish	1	0	0	1	1
Crustaceans	1	1	0	0	0
Mollusks	1	1	0	0	0
Drosophila	2	2	2	2	0
Mosquito	1	0	2	0	0

NOTE: 0: No significant progress.

1: Has been accomplished experimentally (proof of concept).

2: Routine experimental use.

3: Commercialisation sought.

4: Widespread production.

The ability to reprogramme the nucleus of donor cells for successful nuclear transfer appeared initially to require the use of methods that facilitate cell cycle synchrony since only after the donor cells were induced to become quiescent could offspring be obtained by transfer of the donor nucleus to enucleated oocytes. The necessity for quiescence is not as clear today, since nuclei from actively dividing cells have now also been used successfully for this purpose. The ability

to reprogram the donor cells also depends on the species and nuclear transfer procedure. One hypothesis is that differences in timing of embryonic genome activation contributes to differences in cloning efficiency among species. Currently, only oocytes can be used successfully, as they are the only recipient cells that convert differentiated nuclei into undifferentiated stages resembling pronuclei in freshly fertilised zygotes, a step which is essential for the complete development of the reconstructed embryo. How the enucleated oocyte (cytoplast) accomplishes this reprogramming is currently unknown.

At present, propagation of animals by nuclear transfer is inefficient, with an average of less than 10 percent of the embryos resulting in live offspring, although the success rate appears to be increasing with experience. Most of the failures occur during development (most often in the first third of the pregnancy for cattle and sheep) and there appears to be an increased rate of perinatal death relative to normally-conceived offspring. In cattle, at least, the developmental and perinatal problems appear to be as much a function of the *in vitro* culture technology as of the nuclear transfer itself. However, even with this existing low efficiency, there are many potential applications for reproducing highly desired genotypes, including rare or endangered species, household pets, elite sires or dams, breeds with desirable production traits but low fertility, sterile animals such as castrates and mules or transgenic animals that have high value and for which rapid propagation is desirable. Another important application of this technology is in the dissemination of germplasm as embryos and consequent reduction of the associated risk of disease spread. It also is important to note that there are significant differences between cattle and swine in terms of the utility of this technique. In cattle, the ejaculate from a single bull can be used to breed 400 to 500 females in AI programs. In contrast, the ejaculate from a single boar can be used to breed only 10 to 20 females. Thus embryos obtained from NT might be the method of choice for the dissemination of swine germplasm rather than AI. A number of variables influence the success rate of nuclear transfer. These include: species, source of the recipient ova, cell type of donor nuclei, treatment of donor cells prior to nuclear transfer and the techniques employed for nuclear transfer.

## Different Issues of Germiline Modification

A key consideration in development of transgenic animals is ensuring that the gene product of interest is expressed in the correct tissue and at the appropriate level and time. Specifics of how this goal is accomplished vary considerably from one system to another, but some general principles can be elucidated. First, the natural regulatory elements (promoters) for most genes that direct tissue-specific transcription are complex, large and poorly understood. For this reason, well-characterised promoters for other genes are appended to DNA encoding the desired gene product. Second, the expression of transgenes, especially those derived by transfection, is strongly under the influence of control elements in the DNA around the integration site. These positional effects often lead to silencing of the gene of interest (or other genes near the integration site) or, more rarely, to unregulated. They can be alleviated to some extent by the inclusion of sequences, such as insulators or locus-control regions but it is impossible to predict whether a given construct will show the desired pattern of expression after integration. While these effects do not directly affect the safety or utility of those animals that are eventually used, they do introduce considerable inefficiency into the system. A further problem with obtaining correct expression of an introduced transgene is that introduced genes are subjected to silencing by processes including methylation of C residues at CpG dinucleotides, which frequently are found in chromosomal regions important in the regulation of gene expression. Methylation is a major mechanism for turning off the expression of inappropriate genes in somatic cells. Silencing can occur in somatic tissues, but is particularly acute with introduced genes after passage through the germline, where there is widespread DNA methylation at an early stage of embryogenesis. Normal genes in their proper place have signals—in most cases unknown—that reverse the methylation at the appropriate developmental stage. Such signals generally are not present on many commonly used promoter elements, such as retroviral LTR sequences and expression directed by these elements rarely survives passage through the germline. As a natural example, humans carry thousands

of endogenous proviruses that have resulted from retroviral infection of the germline of our distant ancestors; yet only a very few are ever expressed at any significant level. Proviruses based on commonly used murine leukemia virus (MLV) vectors introduced by deliberate infection of the germline suffer a similar fate. Recently, it has been found that vectors based on human immunodeficiency virus (HIV) can be used to efficiently insert genes into the germline of mice and that genes inserted in this way are not subject to silencing following germline transmission. Such vectors promise to yield more efficient and reliable means of generating transgenic animals of many species. Similar vector systems based on the distantly related feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV) also are being developed. Again, methylation-induced shutoff of gene expression is an issue affecting the strategy and efficiency of production of transgenic animals, much less their safety as producers of useful products.

## Selecting Animals

Germline modification remains a hit-or-miss technology and, with most techniques, only a very small fraction of the progeny obtained has the desired properties of expression, copy number and lack of genetic damage. Thus, large numbers of animals must be screened for the presence and copy number of the inserted sequence, for its properly regulated expression, for the ability of this expression to survive transmission through the germline and, finally, for the desired phenotypic characteristics and absence of unintended genetic side effects. Such screening could require several generations of breeding before one can be confident of the absence of recessive genetic damage and the failure rate of the overall process is very high. As nuclear transfer technology improves, techniques requiring direct introduction of DNA into the animal germline followed by extensive screening of progeny are likely to be replaced by much simpler manipulation and selection of cells in culture, followed by recreation of animals with the desired properties directly from the nuclei of the manipulated cells.

## Major Concerns of Germline Technology

There are a number of safety issues that arise as a consequence of manipulation of the germline. These can be divided into several levels of concern: from the animal (or group of animals); to the human handler, recipient or user of the animal or its products; to the human population as a whole; and the environment. All of these levels are discussed here in the context of the technology used; many are presented in more detail in the following chapters.

Introduction of DNA into a cell—whether somatic or germline—is not a well-controlled process and can lead to a number of undesired genetic consequences.

## Genetic Side Effects

Introduction of DNA into random sites in the germline is a mutagenic event that will affect any gene that happens to be at or near the site of integration. The most obvious effect is the disruption of the integrity of a gene into which the insertion occurs. Since a large fraction of the mammalian genome is noncoding DNA derived from various kinds of silenced transposable elements, not all integration events will lead to gene inactivation; however a fraction of animals selected for the presence of a transgene has been found to carry associated genetic lesions. In mice, for example, it has been estimated that about 5 percent of MLV proviruses integrated into the germline have led to mutations of this sort. Direct DNA introduction can lead to numbers of integrated copies at multiple sites, leading to a risk of creating animals with a variety of genetic defects, which should be carefully screened for in the course of subsequent breeding. For example, one of the very first transgenic mouse lines generated, intended to contain an inserted active oncogene, also suffered a lesion that caused a severe recessive developmental limb defect. A number of other examples of insertional inactivation by transgenes introduced into mice are known and this approach has been proposed as a useful technique for mutagenesis. Two additional points should be noted. First, the successfully transfected embryo might have inserted DNA sequences other than

those that express the transgene, so the point of damage can be at a location different from the active transgene. Second, damage of this sort is often (but not always) recessive, so that it can only be detected by inbreeding to derive animals homozygous at the site(s) of the inserted DNA, adding to the complexity of the screening process.

A related effect is the activation of gene expression in the vicinity of the transgene through the action of the introduced promoter elements. This sort of inappropriate activation of expression is the mechanism of cancer induction in animals infected by a variety of retroviruses and it has been well studied as a model for oncogenesis. There are a number of mechanisms by which the expression of genes adjacent to (or even at some distance from) the integration site can be activated, including promoter and enhancer insertion, as well as gene fusion and introduction of elements that stabilise messenger RNA. Indeed, alteration of expression of genes at genome sites far removed from a transgene has been reported in cell lines, apparently due to altered methylation. Whether this effect also occurs in transgenic animals is not known. Activation effects are likely to reveal themselves as dominant mutations that can have a variety of phenotypic consequences, from derailing normal development to causing a high rate of cancer later in life.

## Effects of the Modification

Even if expressed as desired, the genetic engineering itself can often have unexpected effects on the physiology of the engineered organism. One example of such an unwanted effect relates to the xenotransplantation model described above. The galactosyl transferase deficiency in humans, which leads to hyperacute rejection of organ from pigs, also is thought to offer a level of protection against zoonotic infection by enveloped viruses. This effect occurs because the surface proteins of viruses produced by nonhuman cells are also engineered with the same galactosyl-galactose structure found on host cell proteins and are therefore subject to the same potent immune response. This response would lead to the rapid elimination of viruses transmitted

from animals before infection could occur. Pigs that are engineered by knockout of this gene would, therefore, have the potential to transmit viruses, such as influenza, much more readily to human handlers. A related concern is that human cell-surface proteins introduced into animal species as transgenes could render those animals susceptible to human viruses, increasing their risk of disease and providing alternative hosts for the spread of human disease. For example, the human poliovirus receptor (CD155) renders mice susceptible to poliovirus infection when introduced as a transgenic. Also, the human complement-response modifying proteins CD46 and CD55, which are being introduced into pigs to protect xenografts from rejection, also serve as receptors for human viruses—measles and Cocksackie, respectively. Their presence in transgenic pigs not only could render these animals susceptible to infection by the human viruses, but also could provide a new evolutionary pathway for adaptation of pig viruses to human cells. Since the receptors for many other viruses have not yet been identified, the potential for this sort of surprise exists whenever a human cell-surface protein is introduced into another animal species.

## The Marker Genes

Vector constructs used for creating transgenic organisms usually contain genes other than the desired transgene. These genes are typically drug-resistance markers obtained from bacteria, which also can confer resistance to the same or similar drugs on eukaryotic cells. The *neo* gene encoding neomycin phosphotransferase, for example, has been used widely for selecting cells in culture infected with retrovirus or other gene constructs (vectors). In most cases, marker genes remain in vectors used for generation of transgenic (especially knockout animals). While many researchers in the field consider them a relatively harmless convenience, there is a potential for them to cause undesired side effects to the host species (such as aiding in the generation of novel antibiotic resistant pathogens) or the ultimate consumer (such as acting as novel allergens). While their potential for real harm is probably very small, it is difficult, maybe impossible, to prove that marker genes are harmless in consumer products. Such genes are usually



unnecessary to the product itself and can usually be dispensed with by sound experimental design. Their presence raises concerns about the food and environmental safety of genetically engineered animal products.

## Undesired Inserts

In addition to insertion of the correct element at multiple locations, the preparation of material used to generate the transgene (or knockout) might contain additional sequences unrelated (or only partially related) to the one of interest and intent. Even extensively purified DNA fragments derived from plasmids grown in *E. coli* might still contain large amounts of contaminating material derived from the host bacterium. Because such fragments can be heterogeneous in size and sequence, they are difficult to detect in DNA preparations by standard methods like gel electrophoresis.

A particular problem in this regard arises with retroviral vectors, because host cells (especially of mouse origin) often contain large numbers of endogenous virus and virus-like sequences that can, in some cases, constitute a majority of the genomes present in vector preparations. Inadvertent introduction of such sequences into the germline of transgenic animals not only has the potential for creating unintended genetic damage, but also can contribute by recombination to the generation of novel infectious viruses. A well-known example is the inadvertent generation of replication-competent MLV's containing multiple such recombinants during the growth of a vector containing a globin gene. These viruses were highly pathogenic in rhesus monkeys, causing a fatal lymphoma; similar to the disease induced by MLV in mice.

## Potential for Mobilisation

When viral vectors are used for the introduction of genes into the germline of animals, there exists a potential for inadvertent transmission of the gene to other individuals (not necessarily of the same species). This undesirable effect could occur if such an animal were to be infected

with a virus sufficiently similar to the vector to package the vector into virions. For example, if a transgenic chicken were created using an avian retrovirus vector, then infection of the transgenic chicken with any related virus (such viruses are quite commonly found in commercial poultry operations) could lead to the production and release of a virus that could transmit the gene to other animals where its presence and expression might be highly undesirable, such as among wild bird populations. Generation of a replicating virus could occur in the absence of exogenous infection, since many species contain endogenous retroviruses in their genomes that could serve as agents of this kind of mobilisation. For example, in cats carrying murine leukemia virus-based vector constructs, the introduced genes could be mobilised to other cats (or, at least theoretically, to their human hosts) by the endogenous feline leukemia viruses found in most animals. As discussed above, the use of vectors based on HIV has the potential to improve the efficiency of introduction of new genes into the germline of many animal species. Such germline vectors could, in principle, also be mobilised by HIV or a sufficiently close relative. Viruses closely related to HIV are found only in African primates; however, viruses of the same genus (Lentivirus) are fairly common in cats (feline immunodeficiency virus or FIV), cattle (bovine immunodeficiency virus or BIV) and sheep. Despite the distant relationship, FIV has been shown to transfer HIV-based vector constructs from one cell to another, raising a serious concern that similar transfer of genes introduced by an HIV (or any lentivirus) vector could be mobilised among animals infected with these common viruses

A related concern arises with the use of mariner and related transposons (including sleeping beauty) to introduce germline DNA. Related elements have been found in large numbers (14 thousand copies) in the human genome and planaria, nematodes, centipedes, mites, insects and humans suggesting the possibility of horizontal gene flow via transposition among highly diverse hosts. These potentially could be mobilised by the constructs used to transfer mariner-like elements into the germline and their insertion into genes could give rise to

unexpected genetic damage. Horizontal gene transfer also might be mediated by ingestion of DNA.

The possible importance of horizontal gene transfer in eukaryotes is controversial. The most compelling argument for horizontal gene flow in eukaryotes is the ubiquity of transposable elements and endogenous retroviruses in genomic DNA, with no known means for their distribution other than by horizontal gene transfer.

It should be noted that any groups using transposable elements for genetic engineering could express the transposases or hopases in the trans configuration and then delete the gene for these enzymes from the transgene constructs, so that once inserted into the host's chromosome, the element is immobilised. Were this a requirement applied to transposable element vector systems for genetic engineering of animals, the hazards at issue could be minimised or eliminated, so long as active elements capable of mobilising the introduced sequences were not already present in the host animal.

## Creation of New Pathogens

In addition to their potential for mobilisation by interaction with related viruses, transgene sequences also can contribute elements to infecting agents that might modify their ability to cause disease. The donation of drug-resistance genes to bacteria as a consequence of their widespread presence in transgenic livestock is one theoretical example, although the resistance gene would have to be one not found in the environment for the risk of such an event to be significantly enhanced over the natural background. Another example is the possible generation of new retroviruses following recombination between endogenous or exogenous viruses and ones used as vectors for transgenes. This recombination event could result in the provision of new genes or regulatory elements (such as LTRs capable of more efficient expression) that could adversely modify the pathogenic potential of the infecting virus. A recent natural example is the generation, through recombination between an infectious avian retrovirus and a distantly related endogenous element, of a highly

virulent virus, called HPRS-103 or subgroup J avian leukemia virus (ALV) . This virus apparently arose as the result of a single, very rare event, but subsequently has been spread worldwide and has become a source of considerable economic loss to poultry breeders.

## **Issues related to Somatic Cell Nuclear Transfer Technology**

Animals using nuclear transfer from somatic cells have received a great deal of attention recently and it is clear that this technology is fast becoming a practical way to rapidly propagate animals with valuable properties. The DNA genomes of somatic cell nuclei used for this procedure differ in two important ways from those of germline cells. First, they have shortened telomeres at the ends of the chromosomes, a consequence of multiple rounds of cell division in the absence of telomerase, the enzyme responsible for maintenance of telomere length. Since loss of telomere length is the principal mechanism limiting the lifespan of cells in culture, the lack of appropriate-length telomeres might be expected to reduce the lifespan of the newly generated offspring or their progeny, but, surprisingly, telomere length (and lifespan of cultured cells) are restored to normal values following generation of cattle by somatic cell nuclear transfer, even when senescent cells are used to donate nuclei. Second, the methylation state of the DNA of somatic cells is quite different from that of germline cells. Since methylation (at CG sequences) plays a major role in the overall regulation of gene expression, it might be expected that inappropriate methylation states might lead to gross developmental abnormalities in embryos produced by somatic cell nuclear transfer. Indeed, it is possible that the inability of the embryo to properly reprogram methylation and expression is a major cause of the developmental abnormalities often seen in the generation of NT-produced embryos. However, the apparently rapid increase in success rate of this procedure with experience, combined with the fact that animals who survive to adulthood are apparently normal, implies that correct methylation can be restored in NT embryos under the proper conditions. "Correct conditions" might involve having the transferred nucleus in the proper

stage of the cell cycle but this point is controversial. Furthermore, in a direct study it was found that correct methylation and expression levels of several key genes were restored in pig embryos derived from adult cell nuclei. Thus, although nuclear reprogramming is a significant practical issue in the efficient application of this technology, it does not appear to present as insurmountable a barrier as once thought. Apparently the developmental process has a much more robust error-correction system than believed possible a few years ago.

The committee carefully considered the possible concerns that might be raised by use of somatic cell nuclear transfer technology. A few issues regarding animal welfare could be identified, including the possibility of inappropriate gene expression during development due to altered methylation patterns or other developmental problems, such as oversized foetuses, as well as concerns that the widespread application of this technology might reduce genetic diversity of animal populations. However, the effects of cloning are more difficult to anticipate because competing processes are at issue. On the one hand, cloning by its nature produces identical copies of a particular individual, reducing genetic variability relative to what would have been transmitted via conventional breeding. On the other hand, cloning makes it possible to save and utilise genetic variability that would not otherwise be available, for example, the genetic resources from a steer proven to be high performing. The tradeoff between the competing processes is hard to quantify in the absence of simulation modelling with validation from field observations. Whatever the mechanism causing it, loss of genetic diversity could limit the potential for future genetic improvement of breeds by selective breeding or biotechnologic approaches. Further, disease could spread through susceptible populations more rapidly than through more genetically diverse populations.

This latter concern is well documented and several studies illustrate the susceptibility of species with low genetic diversity to infectious disease. Diversity of animal populations, particularly at major histocompatibility (MHC) loci, is a major factor preventing spread of disease, particularly viral disease. Different MHC types recognise

different viral or bacterial epitopes encoded by pathogens for presentation to the immune system. In genetically diverse populations, pathogens can evade the immune response only if they adapt to each individual MHC type following transmission from one individual to another. The requirement for this evolutionary process provides a population of animals with significant protection against the spread of infection. Pathogens can more easily evade host immune response in genetically uniform populations. The consequences of the failure of immunorecognition is illustrated by the deadly epidemics of diseases—such as measles—spread by initial contact between Europeans and isolated New World populations that lacked adequate MHC diversity. Not only could enhanced susceptibility create significant risk for the spread of “new” infectious diseases in “monocultures” of cloned or highly inbred animal populations; it also could create new reservoirs for spread of zoonotic infections—like new strains of influenza—to humans. The seriousness of these concerns, particularly relative to current practice obviously must vary considerably from one type of animal to another and might be alleviated with further technologic advances.

## Conclusion

The technology for modifying the germline of domestic animals is being advanced at a very rapid pace. Indeed, some major advances were reported during the brief period in which this report was being prepared. Although many of the detailed issues discussed in this chapter will no doubt soon become outdated to be replaced by new ones not yet considered, some general issues will remain. In particular, there will always be concerns regarding the use of unnecessary genes in constructs used for generation of engineered animals, the use of vectors with the potential to be mobilised or to otherwise contribute sequences to related environmental organisms and the effects of the technology on the welfare of the engineered animals themselves.

## Genetic Engineering and Animal Welfare

Ideas about animal welfare are shaped by cultural attitudes toward animals and animal welfare has proven difficult to assess because it is so multifaceted and involves different ethical judgments. Animal health and the effects of genetic engineering on it is an area of significant public concern.

Different animal welfare aspects of transgenic and cloning technologies include; their potential to cause pain, distress (both physical and psychological), behavioural abnormality, physiologic abnormality and/or health problems; and, conversely, their potential to alleviate or to reduce these problems. Both the effects of the technologies themselves and their likely ramifications should be addressed.

### Reproductive Manipulations

Superovulation, semen collection, artificial insemination (AI), embryo collection and embryo transfer (ET), are used in the production of both transgenic animals and animals produced by nuclear transfer (NT). Commercial livestock breeders also use many of these manipulations routinely. However, while these procedures do raise animal welfare concerns. These generally are not specific to the production of genetically engineered animals. Few of these procedures have received systematic study from the perspective of animal welfare.

Handling and restraint can be distressful to farm animals but are essential for almost all husbandry procedures, including those involving reproductive manipulation. Certain reproductive manipulations (e.g., the administration of injections to induce ovulation) can cause additional transient distress, as can electro ejaculation.

AI and embryo collection and transfer present a range of animal welfare issues depending on the species used. In cattle, these procedures can be accomplished with minimally invasive non-surgical procedures—the latter under epidural anaesthesia. However, in sheep, goats and pigs these manipulations involve surgical or invasive procedures (laparotomy or laparoscopy) and hence the potential for operative and post operative pain. In poultry species, the hen is killed in order to obtain early-stage embryos. In fish, eggs and milt might be hand-stripped in some species (causing handling discomfort), while in others the males or females must be killed to obtain eggs and/or sperm.

Breeding livestock are valuable but they are subjected to these reproductive manipulations repeatedly during their lifetime. In particular, because of the problems involved in screening microinjected embryos prior to implantation to ensure that they actually are carrying the transgene of interest. Recipient cows might be subject to transvaginal amniocentesis for genotyping; nontransgenic foetuses (or male foetuses) are then aborted and the cows reused as recipients. While this limits the number of recipient animals used, it also raises welfare concerns over the repeated exposure of individual animals to procedures likely to cause pain and distress.

Replacements for or alternatives to, some reproductive manipulations are available. For example, a method has been devised for non-surgical embryo transfer in pigs and ova for some purposes can be obtained from slaughter houses, which eliminates the need for manipulation of live donor livestock females. The use of nuclear transfer to produce transgenic animals could eliminate the problem of repeated elective abortion and reuse of recipient animals, since cell populations with specific genotypes or phenotypes could be selected before embryo reconstruction.



## **In Vitro Embryo Culture**

The development of in vitro embryo culture techniques has provided an alternative to in vivo culture, but ruminants produced by in vitro culture methods, whether or not they are carrying a transgene, tend to have higher birth weights and longer gestations than calves or lambs produced by AI—a phenomenon referred to as large-offspring syndrome (LOS). Surveyed researchers worldwide who use in vitro reproductive technologies with different breeds of cattle and also obtained data from a controlled study of Holstein–Friesian calves. The data showed that only 7.4 to 10 percent of calves produced by AI or ET weighed more than 50 kilograms (kg) and only 0.3 to 4.1 percent weighed more than 60 kg, while 31.7 percent of calves produced by in vitro procedures (IVP) weighed more than 50 kg and 14.4 percent weighed more than 60 kg. LOS animals have more congenital malformations and higher perinatal mortality rates, although the incidence and severity of the effects reported vary widely among studies. The range of abnormalities reported includes skeletal malformations incomplete development of the vascular system and urogenital tract. Immune system dysfunction and brain lesions even when IVP calves are not excessively large, however, they seem to be less viable and more often experience problems like double-muscling, leg and joint problems, hydroallantois, heart failure, enlarged organs and cerebellar dysplasia. A study, found that 3.2 percent of calves born after IVP showed congenital abnormalities as compared to only 0.7 percent of calves produced by AI. Hydroallantois and abnormal limbs and spinal cords were especially prevalent. The mechanism responsible for these effects are unknown, but chromosomal abnormalities and disturbances in the regulation of early gene expression and in communication between the foetus and the recipient mother have been implicated. Cows carrying foetuses produced by IVP show abnormal placental development. Culture conditions are associated with LOS and other developmental abnormalities and changing culture conditions (e.g., by not using foetal calf serum and not co-culturing with somatic cells) can help to decrease the rates of LOS and perinatal mortality. Oocyte quality also might play a role in

LOS and other developmental abnormalities. Because of LOS, difficult calvings (dystocia) can be a problem. The mean rate of dystocia across the five breeds represented 25.2 percent for IVP-produced animals. In the population of Holsteins studied by Kruip and den Dass, dystocia scores were higher (3.05) in IVP than in AI (2.44) or embryo transfer (ET; 2.74) calves, indicating a more difficult delivery in cows carrying IVP foetuses; 14.4 percent of IVP-produced calves died perinatally as compared to 6.6 percent of ET or 6.1 percent of AI calves and 13 percent of IVP calves were delivered by emergency Cesarean section, as opposed to 0.9 percent of calves produced by standard AI techniques. Because of this, it is becoming more common to deliver IVP offspring by elective caesarian section. Again, the number of times that this procedure should be performed on any individual animal during her lifetime is an issue of concern. The selection of older, higher parity cows as recipients is important to decrease the incidence of dystocia. There also is a potential for IVF to have longer-term effects, although detailed data for livestock are lacking. Even though they are heavier at birth and might have enlarged organs, IVP-produced bulls seem to have normal semen quality and heifers show normal reproductive maturation. IVP calves have normal growth rates and slaughter weights. Studies with mice, however, have shown that in vitro manipulation can result in long-term phenotypic changes including retarded growth and abnormal DNA methylation patterns; these changes can be transmitted to the offspring. Intracytoplasmic sperm injection (ICSI) is under development for fertilising livestock embryos and ICSI procedures have been combined with microinjection to produce transgenic animals. A concern is that, since the normal fertilisation method of sperm and egg membrane fusion is bypassed—as is the sperm selection that normally would take place in the female reproductive tract—embryos can be produced from abnormal sperm possibly resulting in abnormal offspring.

## **The Production and Required Quantity of Transgenic Animals**

In producing transgenic offspring microinjection has proved to be an

extremely weak method. Although the success of the method varies by species and gene construct, it has been estimated that less than one percent of microinjected livestock embryos result in transgenic offspring and, of those, typically fewer than half actually express the transgene. A scientist reported efficiencies of between 0 to 4 percent for production of transgenic pigs, cattle, sheep and goats. About 80 to 90 percent of the mortality occurs very early during development, before the eggs are even mature enough to be transferred to the recipient female but postnatal mortality also occurs. Even if an individual does express the transgene, it might not be transmitted to subsequent generations. Approximately 30 percent of transgenic mice are mosaics, which means that they carry the transgene in only some of their cells. High rates of mosaicism are observed in other animals as well. In one study involving transgenic cattle, seven out of eight transgenic founder males produced by pronuclear DNA injection were mosaics. Mosaic founder animals might not pass the transgene to their offspring at all or they might transmit it at a normal or reduced rate. In mice and pigs, the inefficiency associated with microinjection can be compensated for to a great extent by implanting recipient females with multiple embryos. In cattle, however, this can result in difficult births as well as masculinisation of the female offspring (freemartinism) if both a male and a female embryo are transferred. For this reason, embryos usually are cultured temporarily in vitro or in recipient cow, sheep or rabbit oviducts until the stage at which longer-term viability can be established. If cows are used, these developed embryos need to be recovered and then transferred to the recipient animals. Although this technique requires the use of additional animals for the "culturing" stage, it can reduce the number of recipient cows needed by up to 90 percent.

## **Insertional Mutations**

As the inserted DNA can insert itself into the middle of a functional gene, insertional mutations that alter or prevent the expression of that functional gene inadvertently might be generated. It is estimated that 5 to 10 percent of established transgenic mice lines produced by

microinjection have such mutations and it is likely that similar rates would be found in microinjected livestock. Most (about 75 percent) of these are lethal prenatally, but those that are not are responsible for an array of defects in mice, including severe muscle weakness, missing kidneys, seizures, behavioural changes, sterility, disruptions of brain structure, neuronal degeneration, inner ear deformities and limb deformities. Individuals with such mutations can vary enormously with respect to the degree and type of impairment shown. And because many insertional mutations are recessive, their effects do not become obvious until the animals are bred to transgenic relatives. For example, although mice engineered with a transgene for herpesvirus thymidine kinase were normal, their offspring that were homozygous for the transgene had truncated hind limbs, forelimbs lacking anterior structures and digits, brain defects, congenital facial malformations in the form of clefts and a greatly shortened life expectancy. Many of the problems associated with random-site integration, including insertional mutagenesis, could be circumvented by gene targeting, which allows for the controlled integration of transgenes into predetermined loci within the genome. In addition to site-specific transgene insertions, gene targeting also permits the removal (knockout) and replacement of existing genes. However, problems with the expression of inserted genes still can arise, while the phenotypic consequences of knocking out a gene will depend upon the function of that gene.

## **The Expressions of The Gene**

Poorly controlled expressions of the introduced gene can arise many animal welfare problems. Many transgenic animals either do not express the inserted gene or show variable or uncontrolled expression although the percentage of inappropriate expression might be decreasing, as transgenic technologies are refined. It must be noted that earlier experiments with transgenic growth hormone in pigs used metallothionein promoters. Current approaches use more appropriate promoters with greatly reduced abnormalities, although with methods of pronuclear injection, there are still problems and variability. The most frequently cited example of welfare problems arising from

inappropriate transgene expression is that of the so-called Beltsville pigs, which were engineered with a gene for human growth hormone in an attempt to improve growth rate and decrease carcass fat content. Backfat thickness was reduced and feed efficiency was improved, although growth rate was not increased. However, the pigs were plagued by a variety of physical problems, including diarrhea, mammary development in males, lethargy, arthritis, lameness, skin and eye problems, loss of libido and disruption of estrous cycles. Of the 19 pigs expressing the transgene, 17 died within the first year. Two were stillborn and four died as neonates, while the remainder died between two and twelve months of age. The main causes of death were pneumonia, pericarditis and peptic ulcers. Several pigs died during or immediately after confinement in a restraint device (a metabolism stall), demonstrating an increased susceptibility to stress. Similar problems are seen in mice transgenic for human growth hormone. Problems due to growth hormone expression also can be seen when the inserted gene comes from the same or a closely related, species. For example, sheep in which ovine growth hormone inappropriately is expressed are lean but diabetic. In salmonids transgenic for fish growth hormone the largest transgenic fish have growth abnormalities of the head and jaw. Fish with the highest early growth performance are affected the most and have difficulty eating. As a result, growth of these fish is retarded relative to other transgenics at 15 months of age and they die prior to maturation. Thus, the severity of morphologic abnormalities is correlated with initial growth rate, although not all transgenic fish display abnormalities. Transgenic coho salmon exhibit cranial deformities and opercular overgrowth. After one year of development, the overgrowth of cartilage in the cranial and opercular regions of the fish with this atypical phenotype becomes progressively more severe and reduces viability. Further, all F1 progeny were deformed seriously, with excessive cartilage growth in the cranium, operculum and lower jaw and they had low viability. The deformities in the offspring were more severe than those observed in their parents at the same age. Devlin attributed this to the mosaicism between founder and F1 generation, with elevated levels produced in the F1. The best optimal long-term stimulation is achieved in

transgenic individuals that show intermediate levels of initial growth enhancement. As in mice, the genetic background of particular selected strains of farm animals probably is important in determining the severity of the defects associated with the transgene. It is speculated that the deformities found in the Beltsville pigs would have been less severe if the foundation stock had been selected for leg soundness and adaptation to commercial rearing conditions.

## Microinjection Uniqueness

Every transgenic animal produced by microinjection is (theoretically, at least) unique in terms of its phenotype. Pigs transgenic for growth hormone, for example, vary enormously in the number of DNA copies that they have per cell (from 1 to 490) and in the amount of growth hormone that they secrete (from 3 to 949 nanograms per millilitre or ng/ml). Half of pigs transgenic for a gene (*c-ski*) intended to enhance muscle development experienced muscle weakness in their front legs and in general the degree and site of muscle abnormality in these pigs varied considerably from one individual to another. This variability makes the task of evaluating the welfare of transgenic animals particularly difficult, since adverse effects almost are impossible to predict in advance and each individual animal must be assessed for such effects. Researchers describe the difficulties involved in such assessments. In most cases, deleterious phenotypic changes in transgenic farm animals—particularly animals transgenic for growth hormone or other growth promoting factors—have been easy to detect because they cause such gross pathologies. However, more subtle effects also are possible. Growth hormone, for example, has many systemic effects, including effects on the efficiency of nutrient absorption, fecundity and sexual maturation. Growth hormone constructs in salmonids have been shown to influence smoltification gill irrigation, disease resistance, body morphometry, pituitary gland structure, life span and larval developmental rate. Gene insertion and removal also can have effects on behaviours—sometimes subtle. For example, growth hormone constructs in fish have been found to affect swimming ability feeding rates and risk-avoidance behaviour. Some types of knockout

mice also have been found to exhibit behavioural problems, such as increased aggressiveness and impaired maternal and spatial behaviours that are not immediately apparent, but that significantly could affect housing and care requirements.

Sometimes adverse effects are seen only when animals are challenged in some way. The abnormal stress response of the Beltsville pigs, when restrained, is an obvious example. In addition, some problems might not become evident until later in development. Mice transgenic for an immune system regulatory factor, interleukin 4, develop osteoporosis, but not until about two months of age. This emphasises the importance of monitoring the welfare of founder transgenic animals and sometimes successive generations, throughout their lifetime using multiple criteria, including behavioural abnormality, health and physiologic normality. There has been only a limited number of studies of the welfare of transgenic farm animals to date and detailed behavioural studies are particularly lacking.

## **Somatic cell Nuclear Transfer**

Nuclear transfer (NT) is a relatively new process and currently is very inefficient. High prenatal mortality and developmental abnormality, LOS, perinatal mortality and abnormal placentation commonly are reported in cloned cattle and sheep. Most mortality in cloned offspring appears to occur within the first few days after birth, although later mortality also is seen. Health and welfare problems reported in the immediate postnatal period include respiratory distress, lethargy, lack of a suckling reflex, cardiomyopathy, pulmonary hypertension, hydroallantois, hypoglycemia, hyperinsulinemia, urogenital tract abnormalities, pneumonia and metabolic problems. However, such problems are not seen universally in cloned animals; many apparently healthy adult cattle, sheep and goats have been cloned from adult, foetal and embryonic cells. For example in 1999, 10 healthy calves were produced from 100 transferred NT blastocysts; the calves were not exceptionally large, all had a strong suckling reflex and only one required veterinary intervention. A scholar reports that the 24 dairy cows surviving from an original group of 30 cloned cattle are in normal

physical condition for their stage of production, exhibited puberty at the expected age, have high conception rates after artificial insemination and show no clinical or immunologic abnormalities. It is difficult to determine which problems are due to cloning (nuclear transfer) per se, to embryo culture or transfer methods or to some combination of cloning and culture/transfer methods. There is considerable variation among studies in rates of early embryonic death, perinatal mortality, LOS and dystocia. The incidence of these problems actually is sometimes lower in animals produced by NT than is typical for animals produced by IVP. Varying levels of expertise and proficiency with the relevant techniques certainly could be contributing factors. Because of their economic value, cloned animals would be expected to receive a high level of veterinary oversight and intervention, which could contribute to the higher postnatal survival of cloned animals in some studies. In cases where there are neonatal problems, they might resolve within a few days of birth. One possible contributing factor to the high prenatal and neonatal mortality seen in cloned animals is improper epigenetic reprogramming. Cloned animals have abnormal methylation patterns, although the significance of this for embryo development and survival in livestock is unclear. The longer-term effects of cloning and/or improper epigenetic reprogramming on animal welfare have yet to be thoroughly evaluated; as the number of surviving cloned livestock increases, such assessments will be possible. There still is a need for detailed behavioural studies of cloned livestock, since cloning has been shown to result in the impairment of mice in learning and motor tasks, although this impairment is transient. Clones produced by fusion of nuclear donor cells with unfertilised eggs are not identical twins, but "genetic chimeras," since almost all cloned livestock studied to date have mtDNA from the recipient egg but not from the donor cell. Whether or not there are potential adverse effects on health and welfare due to having nuclear DNA from one source and mtDNA from another are unknown, although mitochondria are responsible for important cellular functions and mitochondrial type theoretically could affect relevant production traits as well. Of course, each time normal fertilisation occurs, nuclear genes from the sperm are introduced into a different genetic mitochondrial environment



than existed in the cells of the male providing the sperm, so the mixing of nuclear and mitochondrial genes is ubiquitous in nature. During normal aging, telomere lengths shorten and this phenomenon has been associated with cell senescence. Normal reproductive processes restore telomere lengths in newborns, but there has been concern about whether this same restoration would be seen in animals cloned from adult cells or whether such animals instead will age prematurely and possibly develop health problems usually seen in older animals.