Impact of Transgenic Technologies on Functional Genomics

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Abstract

Gene transfer technologies in mammals are the focus of renewed interest owing to the recent emphasis on analyzing gene function in the postgenomic era. Three important developments in this area include transgenics, gene targeting and nuclear transfer or animal cloning. These technological innovations have enhanced our ability to analyze gene function at the level of the whole organism and have provided the means to modify gene expression. This review discusses the origins and current status of transgenic technologies. Various applications and technologies including chromosome engineering, stem cells, gene traps and modification of livestock are presented. The impact of mouse technologies and genomics on functional analyses is also discussed.

Introduction

Gene transfer technologies in mammals are the focus of renewed interest owing to the recent emphasis on analyzing gene function in the post-genomic era (Galas and McCormack, 2002). As large numbers of genes are identified, linking the sequence of a gene to its biological function has assumed an industrial-scale importance. At present, large scale, rapid methods are used to identify sequence motifs, potential functional domains and possible function. Remarkable insights are further gained by large-scale expression analysis and protein-interaction mapping studies that successfully profile when genes are active during development, the cell cycle and differentiation. These studies are providing information on placing gene products in a particular pathway such as signal transduction transcriptional cascades, transport or metabolic pathways. This advance raises the issue of determining gene function at the level of the whole organism, preferably in a model system with relevance to human biology. The purpose of this inquiry is two fold: one intellectual, aimed at phenotypic assessment of gene function in the life of an organism; and the second technological, aimed at the exploitation of genes and their products for therapeutic or commercial purposes. This review provides a framework for examining how gene transfer technologies contribute to our understanding of genome interactions and advance our

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biotechnological capabilities.

Twentieth century genetics was dominated by the idea of introducing exogenous genetic material into the genome of experimental organisms. The foundation of bacterial genetics lies in the discovery of transformation, conjugation and transduction. A combination of bacterial gene transfer technologies and molecular biology tools resulted in the birth of recombinant DNA technology in the seventies. The progress made in bacterial genetics inspired development of similar methods to study mammalian genetics. Here, mammalian gene transfer technology is broadly considered under three technological breakthroughs achieved during the past two decades: (a) transgenic technology; (b) gene targeting; and (c) nuclear transfer or 'cloning'. In this review, we describe the emergence of these technologies and current advances in the analysis of mammalian gene function.

Historical Perspective

Transgenic Technologies

Several reviews have examined how transgenic methods were developed simultaneously in several laboratories (Scangos and Ruddle, 1981; Gordon, 1993; Hogan *et al.*, 1994; Palmiter, 1998; Galas and McCormack, 2002). Advances in different areas including cell culture, somatic cell genetics, animal virology and mouse embryology culminated in the development of methods for generation of transgenic animals. In the author's (FHR) laboratory, the discovery of the transgenic method was the culmination of a long-standing interest in mammalian gene transfer beginning with early studies in somatic cell genetics. Although somatic cell genetics is better known for its success in gene mapping, it also provided early methods of gene transfers into mammalian cells (Ruddle, 1981). When cells from two different species were fused, the resulting hybrid cells partially lost chromosomes belonging to one of the donor species. Thus, it was possible to establish hybrid cells that retained a defined set of donor chromosomes in the recipient cells. Subsequently, microcells or cell free extracts were used to transfer single or a few chromosomes into recipient cells. Chromosomal fragments of varying sizes were transferred by these techniques and identified by assaying for the transfer of selective markers.

The first demonstration of DNA mediated gene transfer in mammalian cells was reported almost 40 years ago (Szybalska and Szybalski, 1962). This involved development of drug resistant mutant cell lines deficient in hypoxanthine phosphoribosyl transferase (HPRT) activity and the design of a now widely used selection system, named HAT (for Hypothanthine, Aminopterin and Thymidine). Mutant cells (HPRT negative) were transformed with DNA and rare HPRT positive transformants were selected by culturing the DNA treated

cells in HAT selection medium. DNA-mediated gene transfers were not consistently reproducible until the role of calcium phosphate in forming DNA precipitates capable of uptake by cells was fully appreciated (Graham and Van der Eb, 1973). Subsequently, efficiency of gene transfer in rescuing deficient cells in selection media was variously demonstrated (Scangos and Ruddle, 1981).

The efficiency of gene transfer by the calcium phosphate precipitation method was low, at a rate of one in 10^5 cells. In an attempt to increase the efficiency of transfer, exogenous DNA was directly injected into tissue culture cells with the aid of micropipets directed by micromanipulators (Graessmann et al., 1979; Capecchi, 1980). Transformation frequencies obtained by microinjection were as high as 20% (Capecchi, 1980). Such a high efficiency of transformation provided an impetus to consider direct introduction of DNA into the germ line of an experimental organism. Soon, Gordon et al successfully demonstrated genetic transformation and stable inheritance of an exogenous gene injected into the pronucleus of mouse zygotes (Gordon et al., 1980). This feat was rapidly reproduced in several laboratories and for the first time there was a tremendous opportunity for modifying the whole organism by what was termed a "transgenic" procedure (Gordon and Ruddle, 1981). The term 'transgenic' is now extensively used to describe a variety of gene transfer methods in several organisms.

The development of transgenic technology owes much to the convergence of gene transfer technologies with the efforts of many researchers studying early mouse development by culturing of eggs, generating chimeras and manipulating blastula/morula staged embryos (Hogan et al., 1994, Brinster, 1998; Hammer, 1998; Gardner, 1998). Success of the pronuclear DNA injection method of generating transgenic mice was preceded by two competing ideas for transforming the mouse germ line, one based on retroviral infections and the other based on embryonal carcinoma (EC) cells. The feasibility of using retroviral vectors in generating transgenic mice stems from the observation that retroviruses that infect intact, dividing mouse embryos are transmitted as a Mendelian trait to offspring (Jaenisch, 1976). Based on these observations, retroviral vectors were developed to generate transgenic mice (Jahner et al., 1985; van der Putten et al., 1985). Unlike other methods, retroviral vectors have the advantage of precisely integrating a single copy of the vector directly into the genome without the expression of the viral genome in the recipient cells. The inability to obtain consistent, long-term expression of the transgene has undermined the usefulness of retroviral vectors in generating transgenic mice. However, retroviral vectors continue to find applications in gene therapy and gene transfers in avian embryos (Friedmann, 1992; Federspiel and Hughes, 1997; Solaiman et al., 2000).

The idea of gene transfer through EC cells comes from the observation that when injected into mouse blastocysts, these cells contributed to somatic tissues and germ lines of resultant chimeras (Brinster, 1974, Mintz and Illmensee, 1975; Papaioannou et al., 1975). Transfected EC cells were then demonstrated to transmit transgenes through germ lines, but they were limited by their capacity to contribute to germ lines. The lack of reproducibility of original observations by others also diminished the usefulness of this approach. The experiments described below were forerunners of manipulating mouse embryos via embryonic stem (ES) cells, a predominant method now used to generate knockout mice.

Although pronuclear DNA injection remains a common method of generating transgenic animals, the labor-intensive nature of the technique has prompted many to look for other alternatives. Using sperm as carriers for the introduction of exogenous DNA at the time of fertilization is an ingenious idea (Gandolfi, 2000). Sperm are known to bind to exogenous DNA by a poorly understood mechanism. Initial reports of success at generating transgenic mice by this technique led to widespread interest because of its simplicity and applicability to many species (Lavitrano et al., 1989). However, a number of laboratories failed to reproduce these results, thereby diminishing their application (Brinster et al., 1989). In recent years, sperms introduced by intracytoplasmic injections instead of in vitro fertilization have successfully generated transgenic progenies at a rate comparable to that of pronuclear injections (Perry et al., 1999). Although its application has remained relatively uncommon in mice, sperm mediated transgenesis is now an established technique in Xenopus, where no other methods exist for generating transgenic frogs. (Kroll and Amaya, 1996; Amaya and Kroll, 1999; Beck and Slack, 2001).

Gene Targeting

Gene targeting techniques emerged from the desire to study gene mutations in the whole organism and involved developing methods to mutate the mouse genome in cultured cells and transmit the mutation through the mouse germ line (Capecchi, 2001; Evans, 2001; Smithies, 2001; Muller, 1999). Gene targeting was favored over methods to create random mutagenesis, then considered impractical in mice. The possibility of introducing specific mutations was underscored by initial observations coming from gene transfer experiments using drug resistant cell lines described above. The existence of active recombination machinery in somatic cells was inferred by studying the mode of integration of drug resistance genes (Folger et al., 1982; 1985). Genes introduced in cells often recombined and integrated as tandem repeats in the genome at random sites. In an effort to improve the efficiency of gene transfers, flanking sequences were included, which in turn suggested the possibility of recombination at the endogenous locus by homology with the flanking sequences (Luciw et al., 1983; Kucherlapati et al., 1984; Smithies et al., 1985).

The potential of transmitting a modified gene through mouse germ line was realized via development of the ES cell technology (Evans, 2001). Interest in culturing cells of embryonic origin came from the observation that the 129/Sv strain of mice contained testicular teratomas, a mixture of cell types including teratocarcinomas, some of which could be serially transplanted and maintained (Stevens, 1967). Cell lines established from these teratocarcinomas showed a propensity to differentiate into different cell types, typical of early embryonic differentiation (Evans, 1972).

Although the application of EC cells for gene transfer was limited, these initial experiments led to the isolation of ES cells from mouse blastocysts (Evans and Kaufman, 1981). When injected into mouse blastocysts, ES cells were shown to contribute extensively to different tissues including the germ line (Bradley et al., 1984; Evans et al., 1985). Methods to target genes into ES cells were soon developed in several laboratories. A positive-negative selection scheme that allows isolation of cells containing the correct targeting event was developed (Mansour et al., 1988). In this scheme, the integration of the DNA targeting cassette at the endogenous locus by homologous recombination was selected for using the neomycin (neo R) gene /G418 selection system. In contrast, integrations at random sites were negatively selected for using the tk gene. This positive-negative selection scheme is popular in designing "knockout" gene-targeting strategies. Besides HPRT, early examples of knockout mouse include generation of null mutations for c-abl and β_2-microglobulin genes (Doetschman et al., 1987; 1988; Kuehn et al., 1987; Koller et al., 1989; Schwartzberg et al., 1989; Zijlstra et al., 1989). Currently more than 7,000 genes have been analyzed for function by knockout strategies in the mouse. Information on the available knockout mice can be searched at the Jackson Laboratory Transgenic/Targeted Mutation Database (http://tbase.jax.org).

Cloning
Cloning has been widely practiced in plants and lower animals. However, cloning of mammals has become feasible only after overcoming major technical barriers and crossing certain intellectual boundaries (McLaren, 2000; Solter, 2000). The origins of mammalian cloning can be traced back to attempts to develop nuclear transfer techniques or fusion of somatic cells with unfertilized or fertilized eggs using Sendai virus, a fusion agent commonly used in generating somatic cell hybrids (Bromhall, 1975). These experiments were further galvanized by an effort to induce parthenogenetic development and to examine the concept of progressive loss of potential of non-zygotic cells in supporting embryonic development. Although Illmensee and Hoppe (1981) claimed live birth of mice from nuclear transfer of nuclei derived from inner cell mass cells of a blastocyst stage embryo into an enucleated zygote, these results were not reproducible. Nuclear transfer experiments carried out independently in Solter and Surani's laboratories failed to clone mice, but led to the discovery of the imprinting mechanism (McGrath and Solter, 1984; Barton et al., 1984). This discovery demonstrated that non-equivalent contributions from the male and the female pronuclear genomes were essential for embryonic development. The genomes of the male and female gametes undergo differential epigenetic modification, making each other's contribution an essential feature of normal development. A number of imprinted genes have been discovered (Solter, 1998; Ferguson-Smith and Surani, 2001). These studies, although fascinating, designated cloning mice as conceptually difficult task.

However, attempts at cloning continued among other mammals (Willadsen, 1986). Experiments carried out using enucleated unfertilized oocytes (instead of enucleated fertilized eggs), as recipients were successful. In early experiments, donor nuclei were isolated from cleavage stage embryos (Willadsen, 1986; Prather et al., 1987; Collas et al., 1992; Collas and Barnes, 1994; Campbell et al., 1993). The embryonic nature of the donor nuclei was thought to be essential for successful cloning. Followed by success in cloning sheep from nuclei isolated from cultured cell lines, Wilmut and Campbell reported the first mammalian cloning from an adult donor nucleus (Campbell et al., 1996; Wilmut et al., 1997). These experiments carried out in sheep have now been reproduced in other mammals including mice (Cibelli et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Onishi et al., 2000; Polejaeva et al., 2000).

The success of mammalian cloning is due to a switch from a fertilized zygote to unfertilized oocytes as recipients (Solter, 2000). Second, the realization that the relative cell cycle status of the donor nuclei and recipient cytoplasm is important led to improved experimental strategies for egg activation and synchronization. Although cloning efficiency remains poor, the importance of the technique for commercial exploitation of agricultural animals and the biomedical value of therapeutic cloning has become increasingly evident. The nuclear transfer technique has provided a novel route to transgenics and gene targeting in mammals (Schneike et al., 1999; McCreath et al., 2000; Denning et al., 2001; Lai et al., 2002). Nuclei isolated from cells in culture manipulated for transgene expression or gene inactivation are introduced into enucleated eggs. Cloned animals invariably transmit the introduced gene modification. Cells in culture can be screened for appropriate levels of transgene expression prior to nuclear transfer. In animals where the efficiency of transgenic method is poor, cloning provides a powerful alternative.

Current Status

Although the basic methods for generating transgenic and gene targeting in mice have remained remarkably unchanged, a number of strategies have been developed that significantly enhance the efficiency of gene transfer. The first category focuses on conditional alterations in gene expression (Sauer, 1998; Gingrich and Roder, 1998; Nagy, 2000; Mills, 2001; Lewandoski, 2001). The second category focuses on analyzing large genomic regions by devising methods to manipulate large-inserts in mice (Shashikant et al., 1998a; Copeland et al., 2001; Heintz, 2001; Muyers et al., 2001; Mills and Bradley, 2001; Yu and Bradley, 2001).

Condtional Alterations in Gene Expression
Many genes play multiple roles during the life of an organism through embryonic development, differentiation and aging. Either transgenic expression or gene targeting can study the earliest, critical function of the gene. However, the resulting phenotype of such manipulations is often complex and lethal, thereby excluding the study of later functions of the same gene. To overcome these problems, it is necessary to alter gene expression conditionally, in a selective, timely and controlled manner. This can be achieved by devising an inducible gene expression system in which the activity of the target gene is placed under the

control of *cis*-acting elements, which respond to specific inducer or effector molecules. In the absence of the inducer/effector, the state of target gene expression remains unaltered. Change in the level of the target gene expression is achieved by the introduction of the inducer/effector molecule into the system (Lewandoski, 2001).

The induction can be achieved in a single transgenic line, especially when the inducer is a small molecular compound that can be administered to mice by injection or feeding. In contrast, binary transgenic systems were designed when the inducer/effector required for gene expression is a transcription factor or a recombinase (Byrne and Ruddle, 1989; Ornitz *et al.*, 1991; Lakso *et al.*, 1992; Gu *et al.*, 1994). The binary system consists of two transgenic mouse lines, the first of which contains a target transgene whose activity remains unaltered unless crossed with a second transgenic mouse line. The second mouse line contains an 'effector' transgene regulated by a tissue-specific or inducible promoter. The expression of the 'effector' in the second line should not affect the normal mouse development presumably for lack of its target promoter in the second mouse genome. When these two transgenic lines are crossed, the expression of the target gene is altered in those progenies that inherit both transgenes. In some instances, the principles of binary and inducible systems are combined to elicit conditional alterations in gene expression (Lewandoski, 2001).

A variety of inducible gene expression systems are currently in use for transgenic mice (Gingrich and Roder, 1998; Mills, 2001). Inducers such as heavy metal ions (zinc or cadmium), heat shock, antibiotics, steroid hormones or IPTG are used to induce gene expression in transgenic mice. There are several problems associated with inducible and binary transgenic systems. First, the response elements generally lack specificity, as they respond to other activators beside their main inducers, resulting in leaky or uncontrolled basal expression. Second, the inducer/effector may affect expression of endogenous genes other than the transgene, making it difficult to absolutely correlate the resulting phenotype to the induction of transgene expression. Very often, the systemic concentration of the required inducer may have deleterious effects on mice unrelated to target gene expression. Thus, the challenge is to design an inducer that at low concentrations results in dose-dependent induction of high levels of target gene expression by interacting with specific response elements, which in turn are refractory to other inducing factors affecting mouse developmental and differentiation processes.

An early example of inducible transgenic mice is the metal-induced growth hormone mouse (Palmiter *et al.*, 1982). In this experiment, the rat growth hormone gene was placed under the control of a mouse metallothionein promoter. Transgenic mice fed with zinc showed dramatic growth, and the experiment inspired many with the power of transgenic technology. However, the metallothionein promoter also responds to other trans-acting factors, some of which are constitutively expressed, resulting in a basal level of expression, thus limiting its use. The interferon-inducible promoters show rapid and high level induction of transgene expression (Arnheiter *et al.*, 1990). However, in

addition to leakiness of the promoter, interferons evoke diverse physiological responses in mice.

Bacterial Inducible Systems

Two bacterial inducible systems employed in transgenic studies are based upon the *lac* and *tet* operons (Gingrich and Roeder, 1998; Mills, 2001). The *lac* repressor (*lacR*) binds to the *lac* operator sequences and inactivates transcriptional activity of the promoter. The synthetic inducer IPTG relieves this repression, activating transcription by several hundred fold. Thus, an inducible transgenic system can be constructed in which the *lac* operator sequences are placed in the promoter of the target gene. The activity of the target promoter is repressed in the presence of *lac* repressor encoded by *lacI* gene. The activity of *lacI* can be regulated by a ubiquitous or a tissue specific promoter. In the absence of IPTG, the target gene expression is repressed. Feeding transgenic mice with IPTG relieves the repression and activates the target gene. Although the *lac* operator system is tightly regulated in the bacterial system, several problems associated with the mammalian expression have made this system less attractive. More recently, the *lac* operator/repressor system has been thoroughly modified to achieve efficient regulation in mice (Cronin *et al.*, 2001). Some of these modifications involved changes in the *lacI* gene for better codon usage, elimination of cryptic splicing sites, translational block and removal of sequences prone to silencing by methylation. An improved *lac*-operator/repressor based transgenic system may provide several advantages of achieving highly specific and reversible induction of target genes (Cronin *et al.,* 2001).

A second inducible gene expression system is based upon the tetracycline operator/repressor (Gossen and Bujard, 1992; Gossen *et al.,* 1995). In this system, the tetracycline repressor (TetR) binds to tetracycline (or doxycycline) and tetracycline operator (*tetO*) sequences. The DNA binding domain of *tetR* is fused to a transcriptional activator domain of a viral proteinVP16, to create a chimeric activator molecule, tTA. Currently, two versions of the *tet* inducible systems are in use: tTA (tet-off) and reverse, rtTA (tet-on; Gossen and Bujard, 1992; Gossen *et al.*, 1995). In the tTA system, the absence of an inducer results in binding of tTA to the operator sequences resulting in the transcriptional activation of the target gene. In the presence of an inducer, the binding of tTA to operator sequences is prevented thereby inhibiting target gene expression. In the reverse system, rtTA requires an inducer for its binding to the operator sequence. Thus, in the absence of an inducer, rtTA does not bind to the operator, resulting in transcriptional inactivation of the target gene. In the presence of an inducer, target gene expression is achieved. The two systems differ from one another in their kinetics of induction of gene expression. In the tTA system, the target gene expression is prevented by continuous administration of the inducer. Removal of the inducer results in induction of gene expression, which is gradual, depending upon the clearance of the inducer from the system. In the rtTA system, induction of gene expression is achieved rapidly by administration of the inducer, but suppression of gene expression will be slow, dependent upon clearance of the

inducer from the system. A number of modifications to improve the *tet* systems are currently being tested. These improvements are aimed at altering codon usage, promoter context of *tet* operator sequences, transactivation domain and sensitivity to the inducer. Despite concerns about toxicity of long term use of antibiotics and induction kinetics, the *tet* system has found several applications in mouse transgenic studies (Lewandoski, 2001).

Steroid Hormone-based Inducible Systems
Steriod response elements are among the best-characterized *cis*-acting regulatory sequences. Accordingly, inducible transgenic systems based on glucocorticoid, estrogen, progesterone and other ligands capable of interactions with steroid response elements have been used in transgenic mice studies (Gingrich and Roder, 1998; Mills, 2001). A disadvantage of the system is that several endogenous genes carrying steroid response elements may respond to the inducer and endogenous hormone levels could induce transgene expression, resulting in leaky expression or inappropriate transgene expression. One attractive steroid based inducible system uses a ligand binding domain of the progesterone receptor that has been mutated to prevent binding to progesterone (Wang *et al.*, 1994). Instead, the mutated progesterone receptor binds to antiprogestins such as RU486. A further modification of this system is fusion of the mutated ligand-binding domain with the VP16 activation domain and a yeast GAL4 DNA binding domain, creating a chimeric effector molecule, termed GLVP. In the absence of RU486, GLVP remains inactive bound by an ubiquitous heat shock protein, HSP90. In the presence of RU486, GLVP is released from HSP90. GLVP then forms a functional homodimer that is translocated into the nucleus, where the Gal4 DNA binding domain recognizes yeast UAS sequences activating transcription of UAS linked target genes (Wang *et al.*, 1994; Wang *et al.*, 1997; Wang *et al.*, 1999).

Several laboratories have been developing *Drosophila* molting hormone, ecdysone-based inducible systems (No *et al.,* 1996; Albanese *et al.*, 2000; Hoppe *et al.*, 2000). A chimeric molecule comprising the ecdysone receptor linked to the VP16 activator is used to induce gene expression of a target gene linked to ecdysone response elements. In response to ecdysone, the chimeric molecule forms a dimer with the retinoid X receptor and activates target gene expression. To circumvent activation of endogenous genes that are responsive to retinoid receptors, a hybrid receptor containing *Drosophila* and *Bombyx* ecdysone receptor molecules has been generated (Hoppe *et al.*, 2000). The hybrid receptor does not require retinoid receptor for its activity. Several potential inducers from plant sources have been tested for their ability to induce ecdysone receptor activity with the hope that these compounds do not activate endogenous gene expression in mammalian cells (Saez *et al.*, 2000).

Site-specific DNA Recombinase Based Transgenic Systems
Binary systems based on site-specific recombination offer attractive methods for achieving conditional alteration of gene expression in transgenic mice. Cre recombinase from bacteriophage P1 catalyzes deletion or inversion of intervening sequences flanked by direct or inverted repeats of a 34 bp recognition sequence, *loxP* (Sauer and Henderson, 1988). A transgenic line is established in which Cre recombinase activity is placed under the control of a temporal specific or tissue-specific promoter. A second transgenic line is established in which the DNA sequences to be removed are flanked by two *loxP* ("floxed") sites. The two mouse lines are crossed and in the progeny inheriting Cre recombinase and the floxed gene, temporal or tissue specific deletion occurs. To achieve tissue-specific activation of gene expression, the floxed sequences are inserted to disrupt the promoter activity of the gene. Recombinase mediated removal of these sequences brings the necessary sequences in contiguity for transcription and translation to occur. In contrast, for inactivation, *loxP* is placed in regions flanking coding regions of the gene. Removal of *loxP* and the intervening coding region by Cre recombinase results in gene inactivation.

Cre transgenic mouse lines generated by combining various inducible and tissue-specific promoters become valuable resources, since these lines can be utilized by several investigators to alter the expression of different genes. Many interesting Cre recombinase mice are available, for instance Cre recombinases expressed only in spermatocytes during the meiotic phase of cell division, in one-cell embryos or in a few defined brain cells. A periodically updated list of Cre recombinase lines for conditional gene expression is available (http://www.mshri.on.ca/nagy/Cre-pub.html). The advantage of the binary system is that multiple crosses of the conditional Cre line with selected murine lines containing "floxed" genes can be carried out to achieve different types of tissue-specific alterations in gene expression. For example, the same neuron specific Cre deleter line might be crossed with "floxed" genes creating single "knockout"lines of serotonin receptor subtypes. The single "knockout" lines might then be bred to produce double "knockouts" of the serotonin receptors. Depending on the experimental design, this methodology provides a powerful analytic tool and creates an extensive database on gene function (Sauer, 1998; Nagy, 2000; Gertsenstein *et al.,* 2002).

A second site-specific recombinase system used for altering gene expression in mice is based upon *Flp* recombinase from *Saccharomyces cerevisiae*, (O'Gorman *et al.*, 1991). Similar to Cre recombinase DNA recombination is catalyzed between two 34 bp FRT recognition sites. Although initial applications of Flp/FRT transgenics were hindered by technical difficulties, recent modifications made to the system, including engineering a Flp variant that functions at 37°C, and improving conditions for more efficient recombination will find more applications in mice (Buchholz *et al.*, 1998; Dymecki *et al.*, 1998; Schaft *et al.,* 2001; Awatramani *et al.*, 2001).

Manipulating Large Genomic Regions
Transgenic mice produced by conventional manipulation of small DNA constructs by routine subcloning procedures often proved to be inadequate as they failed to contain all the necessary regulatory elements for achieving proper transgene regulation in mice. Furthermore, many genes

and gene clusters are several kb long with regulatory elements scattered at considerable distances from the coding region, thereby making it necessary to analyze large DNA fragments in mice. Three lines of technological developments have addressed these concerns. First, cloning vehicles that hold large-inserts allow examination of large segments of genome by transgenic analysis (Peterson *et al.*, 1998; Shashikant *et al.*, 1998a; Giraldo and Montoliu, 2001; Heintz, 2001). Second, methods to manipulate large-inserts by recombinogenic targeting methods allow functional analysis of large inserts (Shashikant *et al.*, 1998a; Copeland *et al.*, 2001, Muyrers, 2001). Third, using the Cre/*loxP* system, it is possible to engineer chromosomal deletions, inversions or translocations directly in mice (Yu and Bradley, 2001).

Large-insert Transgenics
Large-insert cloning vehicles that hold several hundred kbs of DNA fragments were first developed by adapting the yeast origin of replication and telomeres along with yeast selectable markers. Yeast artificial chromosomes (YACs) can hold up to 2000 kb. Bacterial artificial chromosomes (BACs) containing the F-factor *ori* and P1 artificial chromosomes (PACs) containing P1 bacteriophage replicon that can hold inserts in the range of 300 kb were developed later as additional large-insert vehicles (Burke *et al.*, 1987; Sternberg *et al.,* 1992; Shizuya *et al.*, 1992; Iannou *et al.*, 1994). Although YACs provide a larger cloning capacity and easier insert manipulation, there are significant drawbacks in the use of YACs for transgenic studies. These include poor cloning efficiency and chronic chimerism in most YAC libraries. In contrast, bacterial based vectors, BACs and PACs, are relatively easy to handle and the inserts are more stable and less prone to chimerism. The yeast-bacteria shuttle vector, pClasper, combines the advantages of BACs and YACs, without their apparent disadvantages (Bradshaw *et al.*, 1995). The DNA inserts cloned in pClasper are stable in yeast and are manipulated using the yeast genetic system. Subsequently, the DNA inserts are shuttled into bacteria and then isolated for generating transgenic mice (Bradshaw *et al.*, 1996). YACs can be introduced into mice either by direct pronuclear injection or by ES-cell technology (Lamb and Gearhart, 1995; Peterson *et al.*, 1997). In the latter method, yeast spheroplasts containing YACs are fused with ES cells. Alternatively, YACs are introduced into ES cells by lipofection. ES cells carrying YAC DNA are then injected into mouse blastocysts to create chimeric mice. The inserts from BACs and pClasper are introduced into mice by direct pronuclear injections. Large-insert cloning vectors make it possible to examine large genes and gene complexes; to identify long-acting *cis*-regulatory elements; to create transgenic models that more faithfully reproduce the functional aspects of endogenous loci; to complement known genetic mutations; and finally, to study higher order chromosomal structures such as X-chromosome inactivation.

Recombinogenic Targeting
Molecular sub-cloning techniques based on availability of unique or rare restriction sites are inadequate in cloning and modification of DNA inserts for production of large insert transgenics. In the post genomic era, refinement of functional analysis of genes will require modifications of large inserts. Precise alterations in both the coding and regulatory sequences are essential for understanding the role of specific sequences in gene function. Methods based on homologous recombination can be used to generate DNA constructs for transgenic analysis. In yeast, compared to other organisms, the frequency of homologous recombination is relatively high (Szostak *et al.*, 1983). Transformed DNA (lacking autonomous replicating sequences) invariably integrates into the yeast genome by homologous recombination. Homologous recombination in yeast has been extensively used to create yeast gene knockouts (Kumar and Snyder, 2001). When transformed with linear vectors (containing ARS sequences) that have regions of homology to yeast genes, circularization of the vector is observed by incorporating missing regions of the gene from yeast genome by a 'gap repair' mechanism. This principle has been extended to capture and modify large inserts in pClasper by simple transformation and selection protocols in yeast (Bradshaw *et al.*, 1995; 1996; Chiu *et al.*, 2000).

Unlike yeast, bacterial recombination machinery has been less thoroughly exploited to modify gene inserts for functional analysis. Linear DNA transformed into bacteria is unstable because of its susceptibility to the action of Rec BCD exonucleases (Kowalczykowski *et al.*, 1994; Myers and Stahl, 1994). Two approaches are used to enhance stability of linear DNA for recombinogenic targeting. In the first approach, RecBCD exonuclease deficient strains have been developed into which linear DNA molecules can be targeted (Bubeck *et al.,* 1993; Oliner *et al.*, 1993). In the second approach, linear DNA fragments are flanked by Chi sites (5' GCTGGTGG 3'), which attenuate RecBCD exonuclease activity and at the same time facilitate RecBCD mediated recombination (Dabert and Smith, 1997; Anderson and Kowalczykowski, 1997; Jessen *et al*., 1998). Another approach to targeted modifications of large inserts is to provide a recombination function in bacteria either through a plasmid containing *recA* or activating phage encoded *recE* and *recT* genes (ET system; Yang *et al.*, 1997; Zhang *et al.*, 1998; Lee *et al.*, 2001). A number of applications of recombinogenic targeted modifications for functional analysis, ranging from generating of targeting constructs to reporter gene analysis are now emerging (Copeland *et al.*, 2001; Heintz, 2001).

Chromosome Engineering
Chromosome engineering refers to generation of Cre/*loxP* mediated chromosomal rearrangements in mice (Mills and Bradley, 2001; Yu and Bradley, 2001). Although initial application of Cre technology was directed towards deleting small portions of genes, it was soon realized that Cre catalyzes recombination over large genome segments flanked by *loxP* sites (Ramirez-Solis *et al.*, 1995). A number of chromosomal rearrangements including deletions, duplications, inversions and translocations can be induced by strategically engineering the position and orientation of *loxP* sites in the mouse genome utilizing homologous recombination strategies in ES cells (Yu and Bradley, 2001).

Cre mediated recombination events leading to chromosomal rearrangements can be identified in mice generated through ES cell chimeras by visible markers (for example coat color markers) tagged to *loxP* sites. A typical targeting strategy involves introduction of two *loxP* sites, each flanked by portions of the HPRT gene (Ramirez-Solis *et al.*, 1995; Liu *et al.*, 1998). Cre mediated recombination (in this examples, deleting the DNA between the lox P sites) reconstitutes the HPRT gene. This event is selected by growing ES cells in HAT medium. Furthermore, the 5' *loxP* site is flanked by a tyrosinase gene and the 3' *loxP* site is flanked by agouti gene, which encode different coat colors when inherited independently. Inheritance of both genes in the same mouse identifies a chromosomal rearrangement and results in a mouse with a distinctive, butterscotch coat color (Zheng *et al.*, 1999a). Different combinations of drug selection, or orientation of *loxP* sites can be used effectively to create different types of rearrangements. Creating a large-scale chromosome alteration requires gene targeting in ES cells with 5' and 3' *loxP* cassettes separately. Mice generated through germ line transmission of the manipulated ES cell genome are identified and interbred. The anticipated chromosome rearrangement is then simply identified by following the inheritance of the coat-color pattern. A number of applications of chromosome engineering are reviewed elsewhere (Mills and Bradley, 2001; Yu and Bradley, 2001). Using these strategies, many mouse models resembling human chromosomal rearrangements have been created (Lindsay *et al.*, 1999; Tsai *et al.*, 1999; Lindsay *et al.*, 2001).

Another aspect of chromosome engineering based on Cre/*loxP* technology is the generation of balancer chromosomes (Zheng *et al.*, 1999b). When two *loxP* sites are in the opposite orientation, Cre catalyzes the inversion of the intervening DNA fragment (up to 20-30 cM). This inversion prevents recombination along the length of the chromosome. Thus, inversions can be used to maintain the genomic integrity of a region. These inversions can be designed to function as a balance chromosome by tagging it with a recessive lethal gene. A coat color marker can be added to the inversion chromosome to follow its inheritance in the progeny. The balancer chromosomes are used in large scale ethylnitrosourea (ENU) mutatagenesis strategies, where lethal mutations are screened against a balancer chromosome, for maintenance, mapping and characterization of new mutations (Justice, 2000). In conclusion, recombination based methods have invigorated gene transfer technologies. Most of these methods are at the early stages of development, with their potential yet to be fully exploited.

Functional Analysis

Owing to genetic resources and a range of methods continuously being developed to manipulate the mouse genome, the laboratory mouse has emerged as a pre-eminent model for analyzing gene function in the whole organism. *In vitro* studies, homology based sequence comparisons, and use of invertebrate model systems have successfully identified gene functions. However, there is an increased realization that manipulating genes directly in the mouse is essential for developing models relevant to human physiology and pathology. For these reasons, mouse models will continue to occupy a central position in the analysis of gene function in the post-genomic era.

The genetic approach to ascertain the function of a gene involves creating gain- and loss-of function mutations. These strategies result in opposing phenotypes; permit complementation tests and establish relationships among various genes and their products, thus ordering the sequence of interactions that constitute a genetic pathway. This classical genetic approach has been successfully employed in microbial, plant and invertebrate systems. Although these tenets have not been rigorously adhered to in the mouse system, the technological advances described above will facilitate intense investigations of mouse genetics in the coming decades. An overview of approaches to functional analysis in mouse is given below.

Gain-of-Function Studies
A gain of function mutation is created by expressing extra copies of the gene of interest in transgenic mice. Changes in the level of gene expression can be achieved within its normal spatiotemporal domain when the gene's expression is directed by its own regulatory region. Alternatively, the expression of the transgene can be achieved at ectopic sites with altered temporal sequence, when its expression is directed by heterologous regulatory elements. Conditional expression of the transgene can be achieved by a number of strategies including induction and recombinase systems, described in the preceeding sections. Early examples of gain of function mutations include expression of a growth hormone gene under the control of the metal-inducible, metallothionein promoter (Palmiter *et al.*, 1982). Transgenic mice fed with zinc showed dramatic growth. Currently, there is a large amount of literature on such studies, impacting every field of modern biology including developmental biology, neurobiology and immunology. Models created by these studies have provided critical information on gene function and identified their relevance to many human and animal diseases and abnormalities.

A major concern regarding gain-of-function mutation studies is that the expression of the transgene is subject to chromosomal influences at the site of integration. The chromatin environment in which the transgene randomly inserts is critical in determining the level of expression. Location of the transgene in heterochromatin or in nuclear compartments where transcription is silenced results in a lack of transgene expression. In contrast, location of the transgene in euchromatin or in the nuclear compartment where active transcription is sustained results in expression of the transgene (Gordon, 1993; Jasin *et al.*, 1996). Furthermore, transgene expression is often copy-number independent, suggesting the significance of the local chromatin environment. Transgene expression is also influenced by *cis*-acting sequences located in the vicinity of the transgene integration site. This often results in transgene expression at unexpected spatial regions and/or temporal alterations of expression.

Further understanding of factors influencing transgene transcriptional activity is critical for improving methods by

which consistent transgene expression can be obtained. Characterization of boundary elements, insulators, and silencers will contribute towards the better transgene design (Sun and Elgin, 1999; Francastel *et al.*, 1999). Locus control regions (LCR), the presence of strong enhancers or nuclear matrix attachment sequences (MARS) can direct the formation of a transcriptionally active domain (Grosveld *et al.*, 1987; Cockell *et al.*, 1999). Sequences, which can direct position-independent, copy-number dependent expression in multiple tissues, have recently been identified (Grosveld *et al.*, 1987; Krempen *et al.*, 1999; Blom van Assendelft *et al.*, 1989; Shewchuk *et al.*, 1999; Talbot *et al.*, 1994, Willoughby *et al.*, 2000). However, these sequences have not yet been incorporated into a general strategy for designing transgenes. Alternatively, one can target the transgene to pre-selected sites via the cell-mediated transgenesis approach (Bronson *et al.*, 1996). Strategies can be designed to specifically integrate a single copy of the transgene at a given site by homologous recombination in ES cells. These targeted cells can then be used to generate transgenic mice.

A second problem related to achieving proper transgenic expression is that the regulatory elements could be scattered and/or at a distance from the coding regions of the gene. With the advent of large-insert cloning vehicles and recombination based cloning and modification systems, it has been possible to achieve accurate expression of the transgene by including larger fragments of DNA in the transgene. Large-insert transgenics are less likely to be influenced by local enhancers and in many instances show copy-dependent gene expression. Faithful, copy-dependent expression of the transgene has been demonstrated for tyrosinase (250kb), *myf5* (680kb), *Igf2* and *H19* genes (130Kb) where small insert transgenics for the same genes had previously failed to recapitulate several aspects of endogenous gene expression and regulation (Schedl *et al.*, 1993; Ainscough *et al.*, 1997; Zweigerdt *et al.*, 1997). Large-insert transgenics have allowed examination of dosage effect in mice. Gene dosage can provide useful information about the function of gene. Large-insert transgenic studies have led to the examination of dosage effect of *minibrain* gene in learning defects associated with the Down's syndrome (Smith *et al.*, 1997). A BAC transgenic mouse overexpressing *Zipro1* discerned its role in proliferation of granule cells in the developing cerebellum, where gene targeting studies had earlier revealed no detectable phenotype for the gene (Yang *et al.*, 1999). These recent developments have now established the importance of gain-of-function mutations towards our understanding of gene function.

Loss-of-Function Studies
Both gene targeting and transgenic methods create loss-of-function mutations. Gene targeting techniques have revolutionized our ability to create a variety of defined mutations in a gene, ranging from subtle mutations and chromosomal rearrangements to tissue specific inducible gene inactivation. Over 7,000 gene knockouts have been described in the literature (Capecchi, 2001). The ever-growing list of mouse mutants generated by gene targeting technology can be accessed on the web (http://www.jax.org/resources/documents/imr). Many of the problems associated with gene targeting strategies in interpreting phenotypes have been discussed elsewhere (Muller, 1999). Gene targeting has undoubtedly provided many insights into gene function at the level of the whole organism. One of the early realizations was that the same gene may perform multiple functions at different stages of the development of an organism. Initial frustrations with the inability to analyze adult functions of genes whose mutations result in an early embryonic lethality led to the development of the conditional, tissue specific and inducible knockout strategies described in the previous section. Introducing different types of mutations by gene targeting provides different aspects of gene function. Multiple targeting strategies have been particularly useful in analyzing complex phenotypes. For example, a null mutation in NMDA receptors led to perinatal death, which prevented the study of their role in the adult central nervous system (Forrest *et al.*, 1994). A hypomorphic allele generated by disrupting one of the introns of the NMDA receptor resulted in reduced expression of the protein rather than no protein as in the null. This condition in turn manifested itself in a behavioral phenotype similar to schizophrenia in the mutant mice (Mohn *et al.,* 1999). Additionally, a conditional knockout of the NMDA receptor in the subregions of the hippocampus resulted in a mouse with deficiencies in learning behavior (Rampon *et al.*, 2000).

Gene targeting studies also revealed remarkable genetic compensation and developmental plasticity in the mouse. Null mutations in a number of genes often resulted in very minor or the absence of detectable phenotypes, invoking the possibility that other members of the gene family may functionally compensate for the mutated gene. A comparison of single and combined mutations in the gene families provided information on overlapping function and specialized roles of each member of the gene family. Synergestic interactions among members of the *Hox* gene family have been demonstrated in many studies. For example, mutations in paralogous *Hoxa4*, *Hoxb4* and *Hoxd4* genes individually result in varying degrees of vertebral transformations. Different double mutations showed different degrees of synergism whereas triple mutation resulted in a much larger transformation of the vertebral column, demonstrating cooperative interactions between the family members, not revealed by individual mutations (Horan *et al.*, 1995). Compound mutations often reveal unexpected functions for the genes, for instance, a role for *Hox* genes in mammary gland development became evident only in the mouse carrying triple mutations in the paralogous *Hoxa9*, *Hoxb9* and *Hoxd9* genes (Chen and Capecchi, 1999).

Besides creating mutations in the coding regions of the gene, gene targeting has also been used to create mutations in *cis*-acting elements to determine the role of regulatory elements in the control of gene expression. The significance of relative position among genes and *cis*-acting elements within the gene clusters has been examined for *Hox* complex and globin gene clusters. These studies have revealed many features of gene cluster organization including enhancer sharing, competition and locus control regions (Dupe *et al.*, 1997; Kondo and Duboule, 1999;

Tanimoto *et al.*, 1999; Kmita *et al.*, 2000; Bender *et al.*, 2001; Spitz *et al.*, 2001).

Loss-of-function experiments are carried out in transgenic mice by directing the expression of dominant negative variants, antisense or ribozymes to specific tissues at desired stages of development. Among these, dominant negative mutations have become a powerful alternative to gene knockout studies. The importance of dominant negative mutations was elegantly outlined by Herskowitz (1987). Dominant negative, as the name suggests, is a dominant phenotype, which can be studied, in heterozygous conditions. The basic strategy involves the inhibition of a wild-type gene product by an overproduced mutated variant of the same product. The variant is designed to contain functional subsets of the domains of the parent protein, but either defective or missing in a subset of domains. An overproduced variant forms non-functional complexes with the wild-type protein and some of its targets resulting in inhibition of a specific function. For example, dominant negative variants can be designed to inactivate recognition, conduction or amplification in a signal transduction pathway. These mutants can probe various functions within a protein complex, thus exploring the relationships between each protein (Perlmutter and Alberola-Ila, 1996; Yuen, 1999). Similarly, dominant negative variants can be designed to disrupt protein-protein interactions, DNA-binding or transcriptional activation. Although dominant negative variants can be designed based on biochemical or structural information, how these variants function in a complex cellular environment is often unpredictable. An additional problem lies in identifying promoters that are functional in the same cells as the wild-type gene, but achieve higher levels of variant expression. In spite of these limitations, transgenic mice derived from dominant negative variants have provided useful information on gene function.

The use of antisense RNA strategy to generate loss of function in transgenic mice has recently been reviewed (Erickson, 1999). Antisense strategy involves the design of complementary oligonucleotides that hybridize with the 5' region of the mRNA, thereby inhibiting its translation. Although antisense methods have been successful in plants, fewer published reports are available in transgenic mice. It is believed that antisense expression must be at least 10-20 fold greater than the specific mRNA to inhibit translation. Use of heterologous promoters and design of transgenes, which encode longer antisense RNA are believed to improve the efficiency of inactivating genes in transgenic mice.

Ribozymes, sequence specific endoribonucleases, have great potential to create loss-of-function by cleaving specific RNA molecules. Ribozymes have been successfully used to target and destroy specific viral and cellular RNAs. They are regarded as an important therapeutic tool as antiviral agents. Several studies have used ribozymes in transgenic mice to knockdown the expression of specific target genes (Welch *et al.*, 1998). Two types of ribozymes, hairpin and hammerhead ribozymes, which have specific nucleotide cleavage sites, have been employed in these studies. Although many *in vitro* studies have shown that ribozymes are effective tools in reducing or eliminating gene expression, their application in transgenic mice is somewhat limited. Examples of ribozyme transgenics include β–*2 microblobulin*, *glucokinase,* α–*lactalbumin*, growth hormone, *amelogenin*, and *HIV-1* among others (L'Huillier *et al.*, 1996; Alami *et al.*, 1999; Andang *et al.*, 1999; Luyckx *et al.*, 1999; Thomas *et al.*, 2001). Lack of toxicity and relatively unique specificity make ribozymes a very attractive tool for transgenesis. Transgenic models are perceived as rapid and inexpensive mouse models to study and evaluate ribozymes for their potential as gene therapeutic agents.

Genetic Complementation
Genetic complementation is considered the ultimate proof for function of a gene. This involves introduction of a functional gene in mutant mice that results in the reversion of the phenotype. Both large-insert transgenics and gene targeting methods have been employed in complementation analysis. Many examples of the rescue of a classical mutation in mice exist. For instance, a 250Kb YAC containing the tyrosinase gene faithfully rescued the albino phenotype in a dosage dependent manner (Schedl *et al.*, 1993). A 670 kb YAC containing the human HPRT locus complements an HPRT mutation in the mouse (Jakobovits *et al.,* 1993). A 140Kb BAC containing the wild type *Clock* gene rescued the loss of circadian rhythm in *Clock* mutant mice (Antoch *et al.*, 1997). Complementation by large-insert transgenics is expected to develop into a valuable resource, particularly in conjunction with the large-scale deletions being generated by the previously described chromosome engineering techniques. Recombinogenic targeted modification methods that allow introduction of subtle mutations in the large-inserts will be analyzed in complementation assays to test whether the introduced changes can rescue the mutant phenotype. These types of studies will link both naturally occurring and experimentally induced mutations to observed phenotypes.

Complementation has been tested by gene targeting methods, especially to examine functional compensation of related genes. This strategy commonly referred to as 'knock-in' uses Cre/*loxP* technology to replace one gene/ domain with another. For example, basic helix-loop-helix transcription factors including *Myf5, MyoD, myogenin* and *MRF4* play important roles in skeletal muscle development. But, they are expressed at different times during embryonic development and mice carrying mutations in these genes have different phenotypes. Mutation in the *Myf5* results in defective rib cage formation. *Myogenin* cDNA was knocked-in the *Myf5* locus by homologous recombination, which simultaneously disrupted *Myf5* function. However, a myogenin knock-in mouse had normal rib cage formation, suggesting functional compensation of *myogenin* for *Myf5*'s role in rib formation (Wang *et al.,* 1996). Subsequent studies showed that *myogenin* can substitute for *Myf5* in promoting myogenesis with somewhat reduced efficiency (Wang and Jaenisch, 1997). Thus, some aspects of myogenesis may be unique to a given myogenic factor while related factors may share overlapping functions in specific pathways.

Knock-in strategies can be very useful in determining redundant and distinctive roles played by genes involved

in a specific pathway. For instance, *c-Fos* and *Fra-1* genes are related proteins and are components of the transcription factor AP-1 complex. The two proteins show high homology in their leucine zipper and DNA binding domains and are expressed in partially overlapping domains during mouse development. Knock-in of the *Fra-1* in place of *c-Fos* revealed that *Fra-1* rescues many *c-Fos* dependent functions such as bone cell differentiation. But, *Fra-1* fails to compensate for *c-Fos* in inducing expression of target genes in fibroblasts (Fleischmann *et al.*, 2000). Knock-in experiments can also provide significant insights on structural relationships among evolutionarily related genes. For example, both *Drosophila engrailed* and mouse *En-2* genes when knocked-in the *En-1* locus rescue the *En-1* phenotype in the central nervous system, but fail to affect the limb phenotype (Hanks *et al.*, 1995; 1998). Knock-in of *Hoxd3* into the paralogous *Hoxa3* locus rescues the *Hoxa3* phenotype (Greer *et al.*, 2000). These types of complementation studies assist in defining the functional domains of the gene under study and provide information on compensatory functions among potentially related proteins.

Transcriptional Regulation
Many of the studies described above require an understanding of the transcriptional regulation of developmental and tissue-specific genes. Transgenic mice provide a reliable system for mapping regulatory elements that control spatial and temporal patterns of gene expression (MacDonald and Swift, 1998; Shashikant and Ruddle, 2001). Mapping of regulatory elements that direct tissue specific expression in a developmentally regulated pattern is critical for understanding gene function. These studies establish regulatory pathways that underlie tissue differentiation and provide powerful tools to manipulate developmental processes. A collection of regulatory sequences, with distinct spatial and temporal specificity will be a valuable biological resource. By careful deletion and mutation analysis, it is possible to engineer regulatory sequences that direct gene expression with exquisite specificity.

Cis-regulatory elements are generally identified by reporter gene analysis in transgenic mice (Cui *et al.*, 1994; Gilthorpe and Rigby, 1999). The bacterial β–*galactosidase* gene is the most commonly used reporter, as its expression can be visualized by staining tissues with X-gal. Recently the gene encoding green fluorescent protein (GFP) has been engineered for use in transgenic mice (Ikawa *et al.*, 1998). Fluorescence allows detection of the reporter gene expression by non-invasive methods. Another reporter gene in use is human *alkaline phosphatase*. Bicistronic reporter gene constructs containing two reporter genes are also available (Li *et al.*, 1997). Typically, a reporter gene is inserted in frame with the coding sequence of the transgene that also contains the flanking genomic regions. Alternatively, fragments of DNA surrounding the gene can be cloned upstream of a heterologous promoter, for instance, the mouse *heat shock protein 68* (*hsp68*) promoter, driving a reporter gene (Kothary *et al.*, 1989). Besides their use in *cis*-regulatory analysis, reporter genes are often inserted in the coding regions in gene targeting

experiments to follow the fate of cells in knockout mice. Additionally, reporter genes are now widely used for cell lineage analysis (Spergel *et al.*, 2001).

A confounding problem in regulatory analysis is that the *cis*-acting elements may be scattered over large distances within the genomic loci. Sequence comparisons and computational programs, so effective in identifying coding regions and functional domains of proteins, are at present ineffective in unequivocally identifying regulatory motifs. *Cis*-acting elements are orientation-independent and tolerate sequence degeneracy. Thus, no significant guiding principles based on sequence analysis have emerged. Comparative sequencing and phylogenetic distance based analysis of non-coding, regulatory regions are just emerging (Kim *et al.*, 2000; Hardison, 2000; Sumiyama *et al.*, 2001).

Although *cis*-regulatory elements may be scattered over large distances, the first place to look is in the immediate vicinity of the gene. Typically, several kb of DNA immediately upstream of the translation start sites are examined for regulatory sequences. If the functional elements are not identified in these regions, the next step is to include larger 5' and 3' DNA fragments and all introns for analysis. To identify regulatory elements acting at considerable distance, reporter genes are inserted in the coding regions of large-inserts by recombinogenic targeting methods and the large reporter gene constructs generated are analyzed in transgenic mice (Chiu *et al.*, 2000; Carvajal *et al.*, 2001; John *et al.*, 2001; Reizis and Leder, 2001).

Cis-regulatory regions for a large number of developmentally regulated and tissue-specific genes have now been characterized. DNA fragments as small as 100-200 bp are known to confer reproducible, spatiotemporal patterns of gene expression to the reporter genes in transgenic mice (Shashikant *et al.*, 1995; MacDonald and Swift, 1998). These compact enhancer regions are often multi-component, partially redundant and interdependent, similar to those characterized for viral enhancers in cell transfection systems. In many instances, mutational analyses have defined critical nucleotide sequences essential for the enhancer activities. The identification of the critical sequence has often led to the candidate transcription factors regulating the enhancer activities (Sham *et al.*, 1993). Interactions between the transcription factor and *cis*-acting elements are confirmed by either gain-of-function or gene targeting type experiments thereby establishing components of the regulatory pathways involved in specific developmental processes.

Transgenic analyses of *cis*-acting elements combined with sequence comparisons of regulatory regions among diverse species are providing many evolutionary insights. Genomic regions with a high degree of conservation provide candidates for *cis*-acting elements that can be examined in transgenic mice (Marshall *et al.*, 1994; Aparacio *et al.*, 1995; Morrison *et al.*, 1995). A comparison of non-coding sequence between distant species has often led to the identification of critical elements controlling tissue-specific gene expressions. In contrast, a careful comparison of well-defined enhancer sequences among different vertebrate species has led to identification of subtle variations that could be correlated with changes in gene

expression and phenotype (Shashikant *et al.*, 1998b). These studies are focusing on variations in regulatory regions as the basis for evolution of diverged morphologies among vertebrates. The role of well-defined compact enhancers in mouse development is also examined by gene targeting methods. Deletion or mutation of the enhancer region or its replacement with heterospecific enhancers have been performed to assess the contribution of specific enhancer elements to the expression of the gene and its subsequent phenotype (Gerard *et al.*, 1997; McDevitt *et al* 1997). Both reporter gene analysis and recombinogenic targeting methods have been employed in analyzing regulatory constraints that are critical for clustered gene organizations. For example, long range *cis* effects affecting larger regions of chromosomes, such as X-chromosome inactivation, have been delimited to specific DNA fragments (Lee and Jaenisch, 1997). In summary, the combined use of large-insert transgenics and recombinogenic targeting methods will make regulatory analysis much more rapid and efficient in the near future.

Imprinting and Reprogramming
The phenomenon of imprinting was discovered by nuclear transplantation experiments described in an earlier section. Imprinting provides mammalian development with an additional layer of epigenetic control. Imprinted genes regulate critical aspects of development including placentation, growth, energy metabolism, lactation and behavior (Solter, 1998; Surani, 2001; Ferguson-Smith and Surani, 2001). To date, about 45 imprinted genes have been identified (http://www.mgu.har.mrc.ac.uk). Certain genes are differentially modified in paternal and maternal genomes, resulting in repression of the gene expression in respective genomes. Normal development is possible only when the expression of the gene originates from the unmodified genome of the parent. For example, *Igf2* is repressed in the maternal genome and active only in the paternal genome (Reik *et al.*, 2000). In contrast, *Igf2r* is repressed in the paternal genome and active in the maternal genome in the mouse (Schweifer *et al.,* 1997). Imprinted genes are often organized in clusters and the analysis of the imprinting mechanism has revealed several interesting regulatory elements including differential methylation, silencers, boundary elements, and antisense transcripts (Ferguson-Smith and Surani, 2001). These elements ensure monoallelic expression of imprinted genes. Differentially methylated regions (DMRs) are strongly associated with imprinted genes. Thus, imprinting has emerged as an important model for studying epigenetic regulation of mammalian development.

Whatever the mechanism of imprinting is, it should be reversible in order to account for transmission of paternal or maternal imprints to subsequent generations. Implicit in this requirement is the existence of a mechanism for reprogramming of the genome of gametes (Surani, 2001). It appears that the existing imprints are erased by genome wide-demethylation in the primordial germ cells (PGCs). The initiation of new imprints presumably occurs during gametogenesis. The imprinting mark, once set, cannot be reversed during subsequent development, except in PGCs. Imprinting results in epigenetic asymmetry in the parental genomes at fertilization (Reik *et al.*, 2001; Rideout *et al.*, 2001; Surani, 2001).

A comparison of the genome-wide methylation levels indicates that the overall levels of methylation are low in PGCs, but raises during gametogenesis, with sperm DNA becoming more methylated relative to the oocyte. After fertilization, the paternal genome undergoes marked active demethylation while the maternal genome undergoes passive demethylation. By the blastocyst stage, the zygotic genome is hypomethylated. At subsequent stages, global methylation takes place resulting in an apparently uniform pattern of methylation on both parental alleles by gastrulation (Reik *et al.*, 2001; Rideout *et al.*, 2001).

The epigenetic status of the somatic nucleus is remarkably different from that of the gametes. In cloning experiments, the somatic nucleus transplanted into the unfertilized oocyte must undergo epigenetic modifications and reprogramming in order to initiate the entire developmental process. This involves erasure of the existing epigenetic state and initiation of the embryonic gene expression program. Success at cloning suggests that the oocyte has the machinery required for reprogramming the genome. However, the poor efficiency of cloning and occurrence of several abnormalities in cloned animals suggest reprogramming errors (Dean *et al.*, 2001; Humphreys *et al.*, 2001). Insufficient demethylation, precocious remethylation and erasure of imprints are suspected to result in fetal and placental abnormalities. Future improvements in cloning efficiency depends upon our understanding of imprinting and reprogramming events that confer totipotency to the transplanted somatic cell nuclei. With the success of cloning in the mouse, these problems can be readily addressed in this experimental organism. Delineating components of the reprogramming machinery in the oocyte and PGC represents an exciting area of functional analysis.

Applications

It is clear from the above description that technological innovations, which arose as a result of a desire to manipulate the mouse genome have resulted in fairly sophisticated methods for functional analysis. It is not surprising that these innovations have impacted a diverse field of study with basic, biomedical, and agricultural applications. Some of the significant applications of transgenic technologies are described below.

Finding New Genes: 'Trap Technology'
Although transgenic and gene targeting methods are aimed at studying the function of known genes, the possibility of identifying unknown genes through these technologies was evident very early on (Zambrowicz and Friedrich, 1998; Stanford *et al.*, 2001). Transgene expression is often influenced by local enhancers. As the integration of a transgene into the mouse genome is random, it was estimated that 5-10% of transgenes disrupt the function of endogenous genes (Allen *et al.*, 1988; Kothary *et al.*, 1988). In principle, the identification of the disrupted endogenous gene is possible as the transgene provides a molecular tag. However, several technical difficulties including

rearrangements or deletions induced by the transgene have precluded developing a general strategy for identifying novel genes affecting developmental processes. The idea of trapping genes by random insertion in the genome was further developed in ES cells (Gossler *et al.*, 1989; von Melchner *et al.*, 1989; 1992; Frederich and Soriano, 1991; Korn *et al.*, 1992). The trap vectors, containing reporter genes, can be introduced into ES cells by electroporation or retroviral integration. The expression pattern of the trapped gene is visualized by staining for the reporter gene introduced with the trap vector. The trap vector could be mutagenic and also provide a means to determine the sequence of the trapped gene. ES cell clones where mutation has occurred can be used to generate knockout mice by blastocyst injections followed by germ line transmission and breeding to generate mice that are homozygous for the mutation. The utility of this approach has broad application in the post-genomic era (Zambrowicz *et al.,* 1998).

The trap vectors are of four basic types; enhancer trap, promoter trap, gene trap and polyA trap (Zambrowicz and Friedrich, 1998; Stanford *et al.*, 2001). The enhancer trap vector contains a minimal promoter that is insufficient to activate transcription. When integrated close to enhancers in the genome, the promoter is activated and the reporter gene expression reflects the enhancer that is trapped. Promoter trap vector contains a promoterless reporter or a selectable marker gene, whose activity is dependent upon trapping promoters, by inserting near a promoter or in the exon of an unknown gene. A gene trap vector contains a reporter gene or a selectable marker gene preceded by a splice acceptor. These vectors are expressed when integrated into an intron of an unknown gene, resulting in a fusion transcript containing the 5' exon(s) of the trapped gene. PolyA trap vectors are a more recent addition to the gene trap methods. Here the expression of the selectable marker gene is under the control of a promoter that is active in the ES cells, but lacks a polyA addition signal. Gene expression is achieved by trapping polyA addition signals at the site of integration near the 3' end of the trapped gene. Gene trap vectors require that for selection, the trapped genes should be transcriptionally active in the ES cell. In contrast, the polyA trap vectors trap non-expressed genes as well. Special trap strategies can be designed to identify sub-classes of genes of interest. For example, secretory-trap vectors are designed to identify secreted and transmembrane proteins that are expressed in the ES cells, based on the fact that the β-galactosidase gene activity is abolished in the lumen of the endoplasmic reticulum. Its expression can be assayed only when the signal sequences are acquired by the trap vector resulting in the secretion of the reporter gene (Skarnes *et al.*, 1995). Trap strategies are also employed to identify genes that are expressed in specific differentiation lineages or as a result of induction by external reagents. Libraries of frozen ES cells, each containing a trapped gene and the information on partial sequence of the trapped genes are available (Zambrowicz *et al.*, 1998). With the completion of the mouse genome sequencing projects, the identity of genes mutated in ES cells can be discerned. Mutated ES cells can be used to generate mice for the assessment of function of the trapped gene. Thus trap technology has emerged as a high-throughput random mutagenesis approach in the mouse that promises to simultaneously facilitate the identification of new genes, the study of gene expression pattern and the phenotypic analyses.

Stem Cells and Therapeutic Cloning
The potential of ES cells to differentiate into multiple cell lineages has been known for many years and their use in tissue repair has long been contemplated (Colman and Kind, 2000). Animal cloning by nuclear transfer demonstrated that an adult nucleus can be reprogrammed to produce an entire organism has thrust the field into the forefront (Wilmut *et al.*, 1997). Success at the derivation of the human ES cells has now raised the possibility that ES cells can be derived from individual adult patients, and differentiated into the desired cell types needed for repair of damaged tissues (Thomson *et al.*, 1998). Furthermore, there is an increased realization that adult stem cells isolated from many organs are more plastic than previously thought. Thus, stem cell research and therapeutic cloning have emerged as new frontiers of biological research (Colman and Kind, 2000; Fuchs and Segre, 2000; Thomson and Odorico, 2000; Blau *et al.*, 2001; Lovell-Badge, 2001; Donovan and Gearhart, 2001; Spradling *et al.,* 2001; Reya *et al.*, 2001; Temple, 2001; Bianco and Robey, 2001).

ES cells when injected into a blastocyst contribute to all the tissues of the chimeric mouse. In culture, ES cells are maintained in an undifferentiated state in the presence of the cytokine, leukemia inhibitory factor (LIF; Smith and Hooper 1987; Smith *et al.,* 1988; Williams *et al.,* 1988). When LIF is withdrawn, ES cells aggregate into embryoid bodies, where they differentiate into a variety of cell lineages (Bradley, 1990; Fuchs and Segre, 2000). Different combinations of growth factors direct embryoid bodies to different cell types including adipocytes, erythrocytes, macrophages, vascular smooth muscle cells, oligodendrocytes or astrocytes. With more than 2000 growth factors now cloned or purified, there is tremendous potential to employ growth factor supplements to direct ES cells to differentiate into specific cell lineages. Studies using mouse ES cells provide the necessary groundwork for differentiating human ES cells into diverse cell lineages for future clinical applications (Fuchs and Segre, 2000; Colman and Kind, 2000; Thomson and Odorico, 2000; Donovan and Gearhart, 2001).

The existence of stem cells in adult tissues for the purpose of maintenance of tissue homeostasis has long been known (Fuchs and Segre, 2000; Spradling *et al.*, 2001; Temple, 2001). These are slow-cycling cells, few in number that occupy specific niches within adult organs and are capable of self-renewal and differentiation. Adult stem cells were viewed as restricted in their differentiation capacity and able to regenerate only those cell types for the organ where they were resident. However, the realization that adult stem cells are much more pliable and capable of differentiating into a variety of tissues is more recent (Blau *et al.*, 2001). For example, bone marrow derived cells besides contributing to the blood also contribute to muscle, brain, liver, heart, and the vascular endothelium (Ferrari *et al.*, 1998; Gussoni *et al.*, 1999;

Brazelton *et al.*, 2000; Mezey *et al.*, 2000). Neuronal cells can give rise to blood and muscle tissue (Bjornson *et al.*, 1999; Galli *et al.*, 2000). Skin stem cells can make neurons, glia, smooth muscle and adipocytes (Toma *et al.*, 2001). These studies are rapidly changing the traditional views of adult stem cells. With the current controversy regarding the human embryonic stem cells, adult stem cells are an attractive alternative for clinical applications.

Stem cell potential for repairing tissue injury or curing diseases has been demonstrated in animal models. For example, mouse ES cells differentiated *in vitro* into cardiomyocytes function in intracardiac grafts in mice (Klug *et al.*, 1996). Mouse ES cells derived from glial precursor cells transplanted into a rat with myelin disease, produced myelin in the brain and spinal cord (Brustle *et al.*, 1999). Many stem cell based tissue engineering projects, including bone, skin, cornea and nervous tissues are now under clinical trials (Colman and Kind, 2000). Stem cell based therapies have been envisioned for many neurological diseases including Parkinson's disease, Huntington's disease, multiple sclerosis and for repairing spinal cord injuries (Temple, 2001). Use of stem cells in producing artificial organs by tissue engineering is a newly emerging field (Bianco and Robey, 2001).

Models for Human Diseases

The development of gene transfer technologies for manipulating the genome has considerably enhanced our ability to create experimental models for studying human pathology. Among experimental organisms, the mouse has emerged as a pre-eminent model system for human diseases. The mouse is the experimental organism of choice to generate models for human disorders and abnormalities. No other experimental organism can be manipulated in as many different ways and mouse physiology closely resembles that of human. Recent progress has led to the identification of mouse genes responsible for many naturally occurring disorders, in parallel with mouse models that are being developed by transgenic and gene targeting methods. The number of mouse models available for the study of human genetic disorders and diseases is large and it is a difficult task to produce here a comprehensive list of available models. Mouse models for human diseases can be searched in Mouse Genome Databases and salient features of these models have been reviewed elsewhere (Bedell *et al.*, 1997a,b). The initial studies focused on producing mouse models of monogenic human diseases including Lesch-Nyhan Syndrome, Gaucher's disease and cystic fibrosis (Kuehn *et al.*, 1987; Engle *et al.*, 1996; Tybulewicz *et al.*, 1992; Snouwaert *et al.*, 1992).

The newly emerging chromosome engineering technology promises to generate more complex mouse models with chromosomal rearrangements including deletions, duplications and translocations (Yu and Bradley, 2001; Lindsay *et al.*, 1999; Tsai *et al.*, 1999; Lindsay *et al.*, 2001). Chromosomal abnormalities are a principal cause of many human diseases that produce early abortions and profound developmental disabilities. Translocations are prevalent in many human cancers including leukaemia, lymphoma and sarcoma. Translocations engineered in the mouse could serve as better models for human leukamia (Buchholz *et al.*, 2000, Collins *et al.*, 2000). Chromosomal deletion of 1.5Mb DNA fragment in mice corresponding to human 22q11 results in cardiovascular defects similar to those observed in DiGeorge syndrome (Merscher *et al.,* 2001).

Chromosome engineering and large-insert transgenic methodologies are likely to be increasingly used in the characterization of polygenic diseases. A number of diseases including diabetes, cancer, epilepsy, bipolar disorder, asthma, and obesity are caused by cumulative effects of mutations at multiple genes. The interactions of genes that cause such complex diseases are called quantitative trait loci (QTL). Although many QTL have been discovered, few causative genes have been identified. One example is the analysis of asthma QTL in transgenic mice (Symula *et al.*, 1999). In this study, a panel of YAC's covering 1Mb intervals of human chromosome 5q31. This region contains 6 cytokine genes and 17 partially characterized genes. Each YAC was used to generate transgenic lines that were analyzed for asthma-associated phenotypes. Functional analysis of QTL is perhaps the next major area of application for transgenic technologies in the mouse.

Gene transfer technology will affect human disease treatment in the clinic using gene therapy applications. There are many ongoing clinical trials aimed at alleviating disease by replacing the missing gene product. These studies suffer from inefficient gene delivery vectors that fail to initiate therapeutic levels of gene product, may not be targeted to a specific tissue, or are only active at therapeutic levels for limited periods of time. While these approaches may be supplanted by therapeutic cloning technologies, they likely will find use in cancer treatment paradigms where cancerous tissue is specifically targeted for toxic therapies.

Animal Biotechnology

With the dramatic demonstration of the power of growth hormone transgenics in mice, biotechnological aspirations for creating heavily muscled and lower fat cattle and other livestock became an immediate goal for many laboratories and private companies (Palmiter *et al.*, 1982; Pursel *et al.*, 1989; Ebert, 1998;Ward and Brown, 1998; Murray, 1999; Niemann and Kues, 2000). Improvements in many economically important traits of livestock including rate of weight gain, milk yield and composition, wool characteristics and egg-laying frequency, have been achieved by hundreds of years of painstakingly slow, yet successful selective animal breeding. The potential for transgenic technology to accelerate selection in livestock is great. However, a number of problems associated with the transgenic technology aimed at improving genetic potential have significantly reduced commercial applications in the agricultural industries. The process of pronuclear injection is less efficient in domestic animals than in mice. Some of the problems of the mouse transgenics, such as variability of success at achieving high levels of gene expression, pose even greater barriers for generating successful transgenic domestic animals. The costs are compounded when considering their longer

generation interval and smaller litter size.

The first transgenic sheep and pigs expressing human growth hormone under the control of metallothionein promoter were reported in 1985 (Hammer *et al.*, 1985). The growth hormone and related genes represent the largest class of transgenes transferred into livestock with the aim of altering body composition. Achieving a controlled regulation of growth hormone genes in livestock has been a challenge. In general, pigs and sheep expressing growth hormone genes have been leaner and consume less feed per weight gain, but they suffer from a number of complications as a result of the high, unregulated levels of circulating growth hormone. Growth hormone transgenics are vigorously pursued in many commercial fisheries in about 35 species including salmon, catfish and tilapia. One example is the transgenic salmon generated by the injection of the salmon growth hormone gene under the control of the ocean pout antifreeze protein promoter. The transgenic salmon grow most rapidly during their first year and reach market size a year earlier than commercially grown non-transgenic salmon (Hew *et al.,* 1995). A number of concerns regarding transgenic fish including consumer acceptance and ecological risks to naturally occurring fish remain to be addressed (Muir and Howard, 1999).

Besides growth hormone transgenics, other examples aimed at improving genetic potential of agricultural animals include transgenic pigs expressing insulin-like growth factor 1 (IGF-1) under the control of an avian skeletal α-actin promoter, resulting in enhanced growth and body composition traits (Pursel *et al.,* 1990). Overexpression of IGF-1 in hair follicles in sheep resulted in 17% increase in wool growth at the first shearing in the first generation. For unknown reasons, no further increase in wool growth was observed at subsequent shearing or in the second generation offspring (Damak *et al.*,1996; Su *et al.,* 1998). Transgenic pigs expressing bacterial phytase under the control of the mouse parotid secretary protein promoter were shown to secrete phytase in saliva (Golovan *et al.*, 2001). Salivary phytase was shown to facilitate greater digestion of dietary phytate phosphorus, and reduce the requirement for inorganic phosphate supplements in feeding and reduce fecal phosphorus output by up to 75%. These pigs offer a unique approach to the management of phosphorus nutrition and nutrient run-off pollution a very significant problem for the pork industry.

One area of livestock transgenics that can be found in various phases of commercial development is 'pharming', which refers to the production of pharmaceuticals in milk (Clark 1998; Rudolph, 1999). Promoters of genes that are expressed in mammary glands such as α-, and β-caseins, β–lactoglobulin from several species are used to drive the expression of a transgene for a therapeutic protein to the mammary glands of sheep, goats, cattle, rabbits and pigs. Proteins produced in their milk, often in large quantity undergo appropriate post-translational modifications and retain high biological activities. At least 17 different proteins are produced in five different livestock that include α–antitrypsin, clotting factor VIII, anti-thrombin III and erythopoeitin for kidney dialysis patients. Many of the recombinant proteins produced in milk are now under different phases of clinical trial. This approach may be more economical than large cell fermentation system when producing therapeutic proteins for medical use (Rudolph, 1999).

Another area of biomedical application of transgenic technology is the production of transgenic animals for xenotransplantation (French *et al.*, 1998; Niemann and Kues, 2000). Transgenic pigs expressing human complement regulatory genes have been generated in an effort to overcome hyper acute rejection response (Cozzi and White, 1995). Such transgenic pigs are likely to be used in xenotransplantation to overcome acute shortage of allogenic organs. Using cloning and gene targeting technologies to produce small swine without the enzymes required to display glycosylated peptides that contribute to organ rejection are also under evaluation (Lai *et al.*, 2002). It remains to be seen whether these approaches will achieve their implied benefit since xenotransplanation must be shown to be free from transferring viral disease across species barriers.

Many of the shortcomings of transgenic technologies in non-mouse species are now overcome by cloning. In contrast to the slow application of transgenic technology to agriculture, cloning of animals is already having a discernible impact on agricultural industries. The ability to use a cell-based screening system for expression of the transgene prior to generating genetically modified organisms (GMOs) has resolved a major issue of transgene expression. Fetal sheep fibroblasts were transfected with human clotting factor IX under the control of the sheep β-lactoglobulin promoter. Nuclei from transfected cells that express the transgene were used to clone sheep by nuclear transfer and the resulting adult ewes secreted factor IX in their milk (Schnieke *et al.*, 1997). The cell-based route for transgenesis via nuclear cloning will replace the tedious pronuclear method of producing transgenic livestock. Nuclear cloning is also providing a method for gene targeting in livestock. Until recently, gene targeting was possible only in the mouse because embryonic stem cells in other species were unavailable. However, gene targeting can now be performed in cultured cells and their nuclei can be used to generate knockout animals (McCreath *et al.*, 2000; Denning *et al.*, 2001; Lai *et al.*, 2002). Besides generating transgenic and gene-targeted livestock, the main application of cloning appears to be in extending genetic gains achieved by conventional breeding. Most production traits in livestock are controlled by a relatively large set of genes that have been selected for by planned breeding schemes or sire selection. Cloning offers a direct method to quickly reproduce elite individual animals without diluting their genetic makeup. Another application of cloning that has caught our imagination are attempts to clone endangered species (Loi *et al.*, 2001; Vogel, 2001). Where breeding in captivity has failed, cloning offers an alternative to preserve biodiversity. Nuclei isolated from Gaur and mouflons when transplanted into enucleated eggs of cows and sheep respectively resulted in live births of the endangered animals. Cell banks could be established to preserve diverse germplasm for future cloning of animals when the need arises.

Impact of Genome Sequencing Projects

Transgenic, gene targeting, cloning and stem cell technologies developed during the last two decades have set the stage for mammalian genetics to dominate future decades. The human genome sequencing project in its conception and execution has provided a playing field for mammalian genetics to fulfill this prophecy. The human genome comprises at least 30,000 genes. Biological functions for only a small fraction of these genes are known. While a variety of computational and expression profiling methods have provided valuable insights into the functions of these genes, the necessity of validating these predictions in experimental organisms is now fully appreciated.

The study of gene function, as discussed in this review, involves creating a variety of modifications in genes and their regulatory elements and assessing the phenotypic effect of these engineered modifications. The pre-eminence of the mouse models for studying the monogenic and polygenic basis of complex human physiology and behavior is well appreciated. Gone are the days when mammalian experimental systems were considered slow, cumbersome and expensive. Academic, governmental and private research institutes are rapidly expanding infrastructures for creating and maintaining transgenic mice. Mouse genomic resources include a large number of both naturally occurring and experimentally induced mutations, more than 475 inbred lines, congenic and recombinant inbred strains; extensive chromosomal mapping resources, chromosome substitution strains and radiation hybrid transcript maps (Beck *et al.*, 2000; Nadeau *et al.*, 2000; Avner *et al.*, 2001; Cai *et al.*, 2001; Hudson *et al.*, 2001). Large scale mouse mutagenesis projects are underway throughout the world with goals of identifying novel mutations by phenotype-driven approaches (Justice, 2000; Nolan *et al.*, 2000). The Genome Exploration Group at RIKEN, Japan has obtained a large collection of mouse cDNA sequences that are being used for expression profiling and functional annotation (Kawai *et al.*, 2001). Two mouse genome projects, one public and the other private aim to complete the sequence of the only other mammalian genome, besides human (Hamilton and Frankel, 2001).

With the completion of the human genome sequence project, the functional annotation of genes is expected to take advantage of the available resources in the mouse such as synteny maps for putative common functional regions. This comparative mapping approach will be extended to other species based on segments of chromosome homology or evolutionarily conserved regions of genomes. Application of these methods in different species is likely to identify genetic loci in other mammals where genomes sequencing is not yet a priority. Comparative genomic approaches are also expected to reveal functional identity of many genes and potential regulatory regions buried in the noncoding regions of the genome. Progress in these areas is now occurring rapidly and emphasizes the need for developing more sophisticated tools for gene transfer and functional analysis. The past decade saw rapid advances in gene transfer technologies and the future of these and newer technologies remains unlimited.

Acknowledgements

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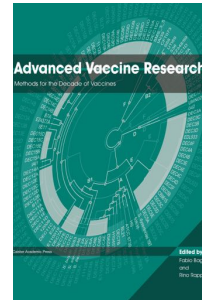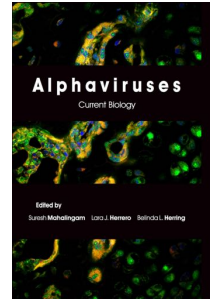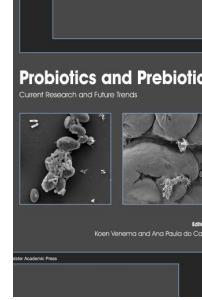
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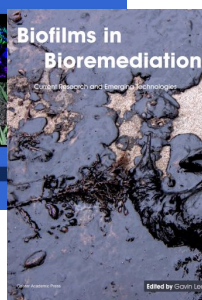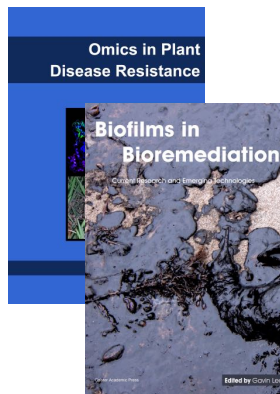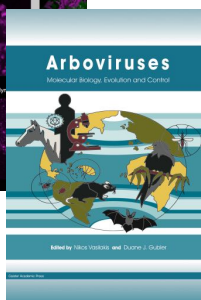
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