

# Perspectives Series: Molecular Medicine in Genetically Engineered Animals

## Biological Insights through Genomics: Mouse to Man

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The increasing pace at which genetic information (genome mapping and sequencing) is being generated as part of the Human Genome Program, is challenging the biomedical community to develop approaches for converting this raw data into meaningful information. The availability of extensive tools for the genetic analysis of the mouse has made it an important substrate in modern biomedical research. Studies in mice have already been enormously effective in deciphering the regulation and function of numerous previously identified genes. This review focuses on ways in which the mouse has been used and speculates on ways it may be used in the future for the discovery and analysis of new genes embedded in the data produced by the genome program. These include: (a) molecular isolation of previously existing mouse mutations that provide insight into human physiology and pathophysiology focusing on obesity; (b) the use of yeast artificial chromosomes to study large regions of contiguous human DNA in mice; and (c) the

use of the mouse to identify genes and elucidate their function based on sequence information generated by the human genome program.

### *Isolation of mouse genes relevant to human disease (obesity): from phenotype to sequence*

Generating and analyzing mutations that affect a particular developmental or physiological process of interest is a powerful and proven approach in *Drosophila* and *C. elegans*. In mice, this forward genetic approach has relied heavily on previously existing mutations identified and collected by mouse geneticists since the turn of the century. The isolation of genes responsible for these previously characterized mutations in mice has accelerated in recent years. A successful conclusion to a positional cloning project is appropriately a cause for celebration, but invariably, is just an important step in a long journey which began with the identification of the abnormal phenotype and approximates conclusion when the pathophysiological link between the identified gene and phenotype is established.

A recent example which has received well-deserved attention from both the popular press and the pharmaceutical industry (1), is the *obese (ob)* gene, discovered in 1949 at the Jackson Laboratory when juvenile obesity was observed in a

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few offspring from the same parents. Weight gain in *ob/ob* mice can reach two- to three-times that of normal and is due almost entirely to white adipose tissue, which occurs at the expense of protein and carbohydrate (2–4). Like most forms of human obesity, *ob/ob* mice are insulin resistant; hyperglycemia and hyperinsulinemia are apparent by 2–3 wk after weaning and contribute to the metabolic derangements that result in a dramatically shortened life-span (5, 6). In addition, greatly elevated levels of adrenocorticotrophic hormone and glucocorticoids play a permissive role in the degree of obesity and insulin resistance which develops in *ob/ob* animals (7–9). A persistent conundrum among all forms of obesity is the extent to which weight gain is caused by overeating vs. reduced energy expenditure (10–12). The relative importance of these two factors is a question not only subject to endless debate, but one that can be surprisingly difficult to answer in many forms of human obesity (13, 14). Hyperphagia is a prominent and early feature in *ob/ob* mice, but when mutant animals were restricted to the exact same caloric intake as their nonmutant littermates (an experiment known as pair-feeding), *ob/ob* animals still exhibited excessive weight gain (15–18). A landmark set of experiments performed by Coleman, in which two animals of different genotype were surgically connected by parabiosis, demonstrated that a circulating product from nonmutant animals could suppress the hyperphagia and weight gain of *ob/ob* animals (19). These experiments, together with analogous ones performed with animals carrying the *diabetes (db)* mutation (a second murine obesity mutation) (20), suggested the existence of a circulating satiety factor which served as the afferent component of a regulatory loop governing adiposity. Peripheral production of the satiety factor was blocked by the *ob* mutation, while the central nervous system response to the satiety factor was prevented by the *db* mutation. From these studies, it appeared likely that the *ob* gene product was a secreted protein produced by adipocytes while the *db* gene product was the hypothalamic receptor to which the *ob* protein bound.

Systematic efforts to identify the *ob* gene were initiated almost nine years ago, at a time when molecular markers on the mouse genetic map were few and far between, and the largest piece of DNA that could be easily propagated in a cloning experiment was 45 kb in length. Crucial elements in the eventual success of this endeavor were the isolation and mapping of thousands of highly informative DNA markers (21, 22), and the development of yeast artificial chromosomes (YACs)<sup>1</sup> (23) as cloning vectors. The former achievement could not have been possible without systematic efforts carried out by genome centers; and the latter achievement has played an essential role for both human and mouse genomic research. When the molecular mapping resources were applied to a breeding experiment in which meiotic crossovers had been generated from 1600 *ob* chromosomes (24–26), the critical region containing the *ob* gene was narrowed to a 650-kb interval. Sorting through this region for expressed sequences eventually revealed that *ob* encoded a novel secreted protein produced by adipocytes, much as was postulated twenty years earlier (26).

Besides identity or lack thereof of the *ob* receptor and the *db* gene product, important questions remain to be answered about how the hypothalamic-pituitary-adrenal axis may regu-

late production of, and response to, the *ob* signal. A human homologue for *ob* has also been discovered; determining the level at which its gene product is expressed in obese human populations will have implications for both understanding normal physiological pathways and developing treatment strategies.

A second mouse gene in which mutations produce obesity is the *agouti (A)* coat color gene, whose name derives from the native language of a South American Indian tribe in reference to a rodent, the *agouti*, whose hairs exhibit a common and intriguing pigmentation pattern. *Agouti* hairs each contain a yellow band on an otherwise black or brown background, a phenotype caused by a precisely timed switch in the type of pigment synthesized by hair follicle melanocytes during normal hair growth (reviewed in reference 27). Several dominant alleles of *agouti*, including *lethal yellow (A<sup>Y</sup>)*, *sienna yellow (A<sup>SY</sup>)*, and *viable yellow (A<sup>VY</sup>)*, produce not only hairs that are entirely yellow, but also a syndrome of juvenile onset obesity, insulin resistance, and premature infertility (28–32). Like *ob*, both hyperphagia and an increased efficiency of energy expenditure are present (33, 34), but unlike *ob*, increased adiposity does not occur at the expense of muscle mass in fat yellow mice, and hypercortisolism is either not present or less pronounced (35–37). An elegant series of transplantation experiments performed by Silvers and Russell demonstrated that *agouti* controlled the production of a paracrine factor. (However, in studies performed by Wolff, parabiosis of *lethal yellow* and nonmutant mice had no effect on weight gain of either partner [38].)

Chromosomal rearrangements have proven to be an essential clue in the isolation of the vast majority of human disease genes identified by positional cloning. Attempts to isolate *agouti* by positional cloning were greatly aided by the recognition that a new *agouti* allele generated at Oak Ridge National Laboratory was associated with an unusual chromosomal rearrangement that placed sequences from the *limb deformity (ld)* gene, normally on proximal chromosome 2, close to the predicted location of *agouti* on distal chromosome 2 (39, 40). Subsequent molecular characterization of this region revealed that *agouti* encoded a novel secreted protein normally produced in the skin, whose presence was precisely correlated with the synthesis of yellow pigment (41–43). Surprisingly, mice that carried obesity-associated *agouti* alleles were found to produce *agouti* RNA in nearly every tissue of the body, in most cases due to the insertion of retrotransposon-like elements that deregulated normal *agouti* expression, a mechanism similar to that which occurs for many proto-oncogenes during tumor formation (44–46). A genetic clue to the mechanism of *agouti*-induced obesity came from the realization that another mouse coat color mutation, *extension* (sometimes known as *recessive yellow*), is caused by inactivation of the receptor for alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) (47). In an otherwise wild-type background, loss of *agouti* function produces black hairs and loss of *extension* function produces yellow hairs. Animals that carry loss-of-function mutations in both genes exhibit the phenotype of the latter rather than the former (in genetic parlance *extension* is epistatic to *agouti*), which suggested that *agouti* protein may act by blocking the ability of  $\alpha$ -MSH to signal through its receptor (48, 49). Biochemical studies have confirmed this hypothesis, suggesting that *agouti*-induced obesity is caused by interference with signaling pathways activated by  $\alpha$ -MSH or related ligands (50, 51).

1. Abbreviations used in this paper:  $\alpha$ -MSH, alpha-melanocyte stimulating hormone; YAC, yeast artificial chromosome.

While obesity is very common and an important contributor to morbidity and mortality in most societies, the majority of human obesity is not caused by mutations in single genes (52). Nonetheless, isolation of the mouse *ob* gene may form a cornerstone in the development of new therapies for human obesity, since activation of the *ob* receptor either by administration of *ob* protein itself, or more likely, a stable agonist for the receptor, could help to augment normal physiologic signals that result in human weight loss. Unlike *ob*, *agouti* does not normally regulate body weight in mice, yet understanding how the *agouti* protein affects pigment cells has led to a new appreciation of the role for melanocortins in regulating energy balance. The effect of both *ob* and *agouti* depends very much on genetic background, and determining the extent to which modifiers of one mutation affect the phenotype of the other may result in additional targets for therapeutic intervention.

#### *Genomic tools in the study of large genes in mice*

Until recently, manipulation and analysis of large genes has been limited by the insert capacity of the standard cloning vectors, cosmids (45 kb) and lambda phage (25 kb). Although cDNA constructs for large genes have been used for the creation of transgenic mice, these constructs lack sequence elements vital to the gene's normal *in vivo* regulation. Even genes with small coding regions may have important regulatory elements located a great distance from the coding region. An example of this is the  $\beta$ -globin locus control region. These regulatory sequences situated > 50 kb away from the 1.2 kb  $\beta$ -globin gene play an essential role in the expression of  $\beta$ -globin *in vivo* (53).

The impediments to the manipulation of large genes have recently been diminished with the development of cloning vectors able to propagate large DNA inserts. Prokaryotic vectors able to accommodate between 80 and 300 kb inserts include P1-phagemids (54), P1 artificial chromosomes (55), and bacterial artificial chromosomes (56). Yeast artificial chromosomes (YACs) (23) propagated in the unicellular eukaryote *S. cerevisiae* can accommodate inserts significantly larger than the bacterial-based vectors and have been indispensable for positional cloning and the development of clone-based maps of the genomes of several model organisms (57–63) as well as entire human chromosomes (64, 65).

Although large insert vectors have mostly been used for genome mapping studies, they are increasingly being used in mouse transgenesis experiments to study properties of large contiguous genomic regions and large genes *in vivo*. The analysis of the entire human  $\beta$ -globin cluster, including distant flanking regulatory elements in YAC transgenic mice, has provided novel insights into how the various genes in this cluster interact with their distant regulatory elements (66, 67). Other examples of large genes that have been studied as transgenes using large insert vectors include the apolipoprotein B gene (P1-phagemid vector) (68, 69) the apolipoprotein [apo(a)] gene (YAC vector) (70) and the amyloid precursor protein gene (YAC vector) (71).

In the case of apo(a), YAC transgenic mice were used to study the regulation of a human gene for which there is no known rodent homologue. Plasma levels of apo(a) have been shown in numerous studies to be an important risk factor for coronary artery disease (72), but little is known about agents that have an impact on apo(a) concentration. In part, this paucity of information stems from the lack of a suitable model sys-

tem; apo(a) is only present in a select group of primates (including humans). The large size of the apo(a) gene (> 120,000 kb) (73) has further hindered its manipulation and analysis. A new substrate for investigating apo(a) was provided by YAC transgenic mice that contain not only the entire apo(a) structural gene, but also more than 60 kb of 5' and 3' flanking DNA (70). Although mice do not contain a homologue to the human apo(a) gene, the assumption that the correct response to cis-regulatory elements of the human gene will occur in the foreign environment of the mouse is supported by the correct tissue-specific expression (liver) of the human apo(a) transgene. Two of the physiological factors speculated to regulate apo(a) expression in humans are sex steroids and acute phase inducers, but studies addressing the role of these factors in humans have produced contradictory results. The apo(a) YAC transgenic mice have allowed investigators to address the *in vivo* role of these factors in environmentally and genetically controllable situations attainable in mice but not in humans. These studies reveal that apo(a) gene expression is negatively regulated at the transcriptional level by sex hormones and is not affected by acute phase inducers. Thus, the ability to isolate a large contiguous segment of the human genome and introduce it into the mouse genome has been key in the development of an experimental substrate to analyze apo(a) expression and its regulation.

#### *The mouse and the Human Genome Program: a substrate for gene discovery*

Since the inception of the Human Genome Program an astonishing amount of information has accumulated. Much of this has been in the form of high quality maps of the human genome (74) based on genetic and anonymous DNA markers, clone-based maps (75), and expressed sequence information. As part of the cataloging of expressed sequence tags obtained from cDNA sequencing, it is likely that in the next 5 yr > 90% of all human transcripts will be partly sequenced (76). Presently, genomic sequencing of model organisms such as *Drosophila*, *C. elegans*, and *S. cerevisiae* are well underway, with completion dates projected within the next decade. Concurrent with these studies, there is an accelerating effort being applied towards sequencing the human genome. Thus, the human genome program which began less than eight years ago is off to a rapid start and its completion, as defined by sequencing of the entire human genome, should occur early in the next century.

Once the sequence of the human genome is complete, a major challenge for the scientific community will be the conversion of anonymous map locations and strings of nucleotides into genes whose functions will have implications for biology and medicine. The task of gathering meaning from the genomic sequence of humans will be especially challenging since the human genome is hundreds of times bigger and more complex than model organisms such as *E. coli* or *S. cerevisiae*. For example, even though the biochemistry of splicing has been extensively studied, assigning splice sites unequivocally to genomic sequence without prior knowledge of gene structure from cDNAs or other technologies is beyond our abilities at present. A similar situation exists for regions such as promoters and enhancers that do not code for protein, but whose ability to bind gene regulatory proteins is critical for normal development and differentiation.

A first line in the conversion of sequence information into

genes will clearly involve the tools of informational sciences. This will include looking for open reading frames, splice sites, and other sequence motifs in the human sequence and comparing this to sequence information available for model organisms. Computational sequence analysis is powerful and will become more so with time, but it seems likely that, for the foreseeable future, in vivo and in vitro studies will be required to understand the function of genes that are predicted to exist based upon computational analysis of DNA sequence.

Experimental analysis of sequence data generated by the human genome program is likely to extensively utilize the mouse for a variety of reasons. A high degree of evolutionary relatedness exists between mice and humans, resulting in conservation of genomic organization and regulatory elements (22). Comparison of human sequences with regions syntenically conserved in the mouse genome will provide important clues to define coding regions, exon/intron boundaries, and *cis*-regulatory elements. In addition, transgenesis and gene-targeting technologies may be used to study gene candidates by determining whether over-expression or targeted inactivation yields a detectable phenotype.

One recent tool developed by the genome program that should contribute to the discovery of new genes and the deciphering of their function is the creation of in vivo libraries of regions of the human genome in mice (77). These libraries consist of overlapping YACs from defined segments of the human genome propagated in a panel of transgenic mice. The term "library" has historically been used in molecular biology to describe collections of clones representing part or all of a complex genome propagated in simple organisms such as yeast or bacteria. In the context of in vivo libraries, this concept has now been extended to indicate the propagation of cloned segments of the human genome as a collection of transgenic mouse strains.

The large size of the inserts (100–1000 kb) contained in the YAC transgenic mice makes it likely that: (a) genes, together with their regulatory elements will be contained intact, and (b) that the transgenes in members of the in vivo library will include several genes. These mice may thus be called "transpolygenic," referring to the likely inclusion of several human genes in the transgene. Recently an in vivo library of the Down's syndrome region of chromosome 21 has been created (77), which contains over 2 Mb of contiguous DNA from the region in a panel of 26 different lines of transgenic mice containing eight different large transgenes. Such a library has a variety of uses. As this region of human DNA is sequenced, open reading frames that potentially represent transcription units will appear. A straight-forward assay to determine whether these open reading frames encode transcription units is to look for expression of the human sequence in a line of transpolygenic mice containing that particular human sequence. The presence of the human transcript can be sought in various tissues of adult animals or by in situ hybridization to embryo whole mounts or sections. Analysis of the hybridization results will tell not only whether the human sequence encodes a transcript, but can also provide a description of the expression site(s) of the particular human gene during different stages of development in the whole organism.

In addition to using members of the in vivo library to both define genes originally identified through sequencing of the human genome and to study their expression, careful phenotypic analysis of these animals may provide clues to the func-

tion of the newly identified genes. In the case of the in vivo library of the Down's syndrome region there are very specific phenotypes that can be sought out. This is because an extra copy of this region in humans results in Down's syndrome with its associated phenotypes (78–80). Mouse chromosome 16 carries nearly all of the genes whose human homologues produce Down's syndrome when present in an extra copy. Remarkably, mice trisomic for chromosome 16 share many features with individuals trisomic for human chromosome 21 (81, 82). Hence, the increased dosage of the Down's syndrome region of chromosome 21 in transpolygenic mice should result in distinct phenotypes and therefore may assist in identifying genes responsible for distinct components of the syndrome.

### Conclusion

Much as the decoding of ancient written languages has provided progressively deepening insights into extinct cultures, completion of the Human Genome Project will provide scientists with an essential infrastructure for probing deeper into the mysteries of human biology. Through positional cloning and recently developed approaches for genetic manipulation of the mouse, researchers have already successfully applied information generated by the human genome program to the discovery of new genes with medical relevance to humans. The reagents and methodologies currently being developed for genetic analysis of the mouse will be important in allowing us to derive meaning from the sequence information generated by the Human Genome Project. However powerful current technologies are, further improvements are clearly still required if we are to keep pace with the speed at which sequence information is generated by this large undertaking. Nevertheless, the data generated by the genome program, coupled with development of tools for converting this data into information with biological content will enable practitioners of the various medical disciplines to rapidly realize the promise of the massive effort underway to map and sequence the entire human genome.

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