Perspectives in Diabetes

Transgenic Approaches to the Pathogenesis of NIDDM

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The pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM) involves complex interactions between multiple physiological defects, both genetic and acquired. The application of transgenic technology to create animal models that address questions concerning NIDDM (and obesity) is a very recent development that is now gaining rapid momentum and receiving deserved attention. In general, transgenic methods afford new opportunities to alter the site or level of expression of functional genes in vivo, to transfer novel foreign genes into animals, to prevent the expression of specific genes, or to replace genes with specific genetic variants. Two general approaches can be applied: 1) conventional transgenics, the transfer to and expression of new genetic information in animals; and 2) gene targeting, the disruption or replacement of specific endogenous genes. Recent transgenic initiatives have provided important insights into 1) the mechanism of glucose-stimulated insulin secretion and the role of potential defects in this system, 2) the regulated expression of genes that control hepatic glucose production, 3) the role of specific molecules that mediate the actions of insulin, and 4) the elucidation of factors that contribute to in vivo regulation of energy balance and body composition. Emerging transgenic strategies should have a dramatic impact on future efforts to assess the function of newly identified molecules implicated in the regulation of in vivo glucose homeostasis and to determine the roles of candidate loci or specific mutations uncovered during the search for new NIDDM susceptibility genes. Diabetes 43:1394-1401, 1994

Non-insulin-dependent diabetes mellitus (NIDDM) is a prevalent and heterogeneous syndrome that results from variable impairment of insulin secretion and insulin action (1,2). In Europe and North America in particular, obesity is an additional common disorder that is undoubtedly linked to increased NIDDM susceptibility (1). It is also important to recognize that genetic factors strongly contribute to the predisposition to develop NIDDM (3), and that other acquired factors, including diminished exercise and the potential adverse effects of even mild degrees of hyperglycemia (glucose toxicity) on insulin secretion and action (4,5), are likely to play important roles.

Given the multitude and complexity of pathophysiological disturbances that are associated with the presence of the NIDDM syndrome, it has been difficult to determine which defects are primary versus secondary (which are causative versus less important phenomena that are only consequences of the altered metabolic milieu). The coexistence of multiple separate defects also precludes an assessment of the relative importance of the individual components. A number of genetic rodent models, such as ob/ob mice and Zucker fatty (fa/fa) rats have been extensively studied to gain further insight into the pathogenesis of obesity and NIDDM (6). However, with the exception of the "yellow obese mouse" (KK-A''), the underlying genetic defects in these cases have not yet been identified (7). These disease models also are limited by the fact that multiple cellular and physiological defects may coexist and by the presence of additional phenotypic alterations, such as increased levels of glucocorticoids, that are not typically apparent in the human syndrome.

The application of transgenic techniques allows investigators to selectively perturb a complex in vivo system by 1) redirecting the site and/or level of expression of functional genes, 2) introducing novel foreign genes into laboratory animals, 3) preventing the expression of a desired gene, or 4) replacing an existing gene with a specifically altered genetic variant. Using these approaches, the physiological functions of specific gene products can be better assessed. Moreover, selective physiological defects can be created and studied to determine their potential contribution to a complex syndrome like NIDDM. Once created, transgenic animal models may also be useful to assess the efficacy or determine the mode of action of potential new therapeutic agents.

The use of transgenic technology for the study of immunologic dysfunction and the pathogenesis of insulin-dependent diabetes mellitus is now well established and has been recently reviewed elsewhere (8,9). In contrast, the application of transgenic technology to questions concerning NIDDM and obesity is a more recent development that is now gaining rapid momentum and receiving deserved attention. In this perspective, I will briefly review current transgenic techniques and potential strategies that may be applied to the study of the pathogenesis of NIDDM. In addition, I will describe several examples of recently reported transgenic experiments involving modulation of insulin secretion, he-

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NIDDM, non-insulin-dependent diabetes mellitus; PCR, polymerase chain reaction; ES, embryonic stem; IAPP, islet amyloid polypeptide; MODY, maturity onset diabetes of the young; MCK, muscle creatine kinase; IRS-1, insulin receptor substrate 1; EGF-I, insulin-like growth factor 1; GCR, glucocorticoid receptor; BAT, brown adipose tissue; UCP, uncoupling protein; GPDH, glyceraldehyde-3-phosphate dehydrogenase; QTL, quantitative trait loci; YAC, yeast artificial chromosome.
**CURRENTLY USED TRANSGENIC TECHNIQUES**

**Conventional transgenics.** Transgenic mice are now routinely produced by microinjection of a linearized DNA construct into the male pronucleus of single cell mouse zygotes isolated from superovulated pregnant females. Transgene constructs may first be tested by expression in transfected cultured cells. After a brief period in culture, injected zygotes are transferred to the oviducts of reciprrent pseudopregnant female mice. Genomic DNA is isolated from tail biopsy material obtained from pups and screened for the presence of the transgene. Transgenic founder mice or their offspring can be tested for expression of the transgene or phenotypic consequences. Thereafter, hemizygous or homozygous transgenic lines are established and characterized further.

**Gene targeting.** Rather than introducing additional genes via the use of conventional transgenic techniques, gene targeting allows for the disruption or replacement of endogenous DNA sequences through the process of homologous recombination (25). To date, gene targeting has primarily been used to create "knockout" mice with null alleles; however, technology now exists for engineering animals in whom specific loss- or gain-of-function mutants have replaced endogenous genes or in whom the knockout is tissue-specific (26; see FUTURE DIRECTIONS).

The elimination of normal mouse genes necessitates construction of a targeting vector containing homologous DNA sequences that are disrupted or contain a deletion. Replacement vectors are designed to result in recombinant alleles in which a portion of the endogenous gene has been replaced by mutated genomic sequences (Fig. 2). Insertion vectors are designed to undergo recombination that results in complete insertion of the vector so as to disrupt the gene while retaining all endogenous sequences, thus creating a partial gene duplication (27). The targeting vector is introduced into pluripotent cultured mouse embryonic stem (ES) cells (25,28–30), followed by selection of correctly targeted clonal cells. A low relative frequency of targeted cells versus cells that have undergone random vector integration has led to the development of screening strategies that include the use of selectable marker genes as illustrated in Fig. 2. ES cells that contain the desired recombinant locus can be identified by Southern blot or PCR analysis (27). Recent studies strongly suggest that targeting frequency in ES cells is enhanced by using vectors derived from isogenic DNA (the 129 mouse strain that ES cells are also derived from) and by increasing the length of homologous sequences within the targeting vector (31–33).

After in vitro manipulation and gene targeting, ES cells can be incorporated into preimplantation mouse embryos to form chimeras by microinjection into morulae or (most
TARGET GENE

TARGETING VECTOR → HOMOLOGOUS RECOMBINATION

TARGETED ALLELE

FIG. 2. Gene targeting via homologous recombination. The most commonly used gene-targeting approach involves the design and construction of a replacement vector (R) that contains sequences homologous to the target gene locus (A). Typically, a proximal coding exon (in this case exon 2) is mutated (replaced or interrupted) by a neomycin resistance gene cassette (NEO), which can be used to select for the presence of the vector (with CRE) after electroporation of cultured ES cells. A herpes simplex virus thymidine kinase (TK) gene cassette is often included in the vector to help distinguish between random integration (common) and homologous recombination (uncommon). Additional selection of ES cells with gancyclovir will specifically kill cells after random integration, where the TK cassette is retained; cells with the correctly targeted allele (C) that have lost the TK cassette will survive. Targeted ES cells are introduced into blastocysts by microinjection followed by the generation of chimeric mice and subsequent germ-line transmission of the targeted allele to progeny.

SPECIFIC TRANSGENIC PERTURBATIONS
Modulation of insulin secretion. A number of transgenic experiments have contributed to our understanding of the basic mechanism of glucose-stimulated insulin secretion and the role that altered insulin secretion plays in the development of NIDDM. Beginning as early as 1986, Selden et al. (35) and Bucchinì et al. (36) were able to demonstrate that a heterologous (human) insulin gene could be appropriately expressed and regulated in the islet β-cells of transgenic mice. In one case, normal levels of insulin mRNA were preserved as a consequence of reduced endogenous insulin gene expression, providing further evidence that insulin feeds back to regulate the expression of its own gene. Additional experiments showed that posttranscriptional regulation may also act to compensate for the superphysiological insulin gene expression (37). By introducing 30 copies of the human insulin gene, Marban et al. (29) were able to override this effect and achieve modest (two- to fourfold increased) hyperinsulinemia. Although younger mice were mildly hypoglycemic, older mice developed glucose intolerance and hyperlipidemia (38). These findings support the hypothesis that hyperinsulinemia might be a primary event leading to increased hepatic lipogenesis and the secondary development of insulin resistance (39).

The mechanism by which proinsulin undergoes proteolytic processing and subsequent secretion via regulated versus unregulated pathways has been addressed by transgenic expression of a mutant human insulin gene (Asp-B10) (40) and by the demonstration that ectopic expression of growth hormone in transgenic β-cells results in its appropriate targeting for secretion via the regulated pathway of insulin secretion (41). Further studies of this type may help to explain the relative increase in proinsulin secretion that is evident in patients with NIDDM.

Recent excitement concerning the potential pathophysiological role of GLUT2 glucose transporter downregulation in islets from diabetic animals (42) has been somewhat tempered by the finding that expression of oncogenic H-ras in transgenic islets resulted in a marked decrease in GLUT2 levels without affecting glucose-stimulated insulin release or in vivo glucose homeostasis (43). A similar controversy, the potential role of islet amyloid polypeptide (IAPP) or amylin, has also been recently approached using transgenic mice. Although preliminary data reported by Hoppen et al. (44) suggest that overexpression of human IAPP in transgenic islets can lead to impaired glucose tolerance, Fox et al. (45) were unable to detect islet amyloid or hyperglycemia in similar mice that were characterized by a fivefold increase in blood IAPP levels. Similar results were obtained by de Koning et al. (46), although they were able to detect accumulation of nonfibrillar human (but not rat) IAPP in islet perivascular spaces. This supports the notion that species-specific factors involving IAPP itself and/or other molecules may be required for frank amyloid deposition.

In contrast to the controversy that surrounds GLUT2 and IAPP, a consensus of opinion has emerged concerning the important role of the high-Km islet/liver hexokinase, glucokinase, in β-cell glucose sensing (47). Although the discovery that glucokinase gene mutations can cause maturity-onset diabetes of the young (MODY) is the paramount basis of this knowledge (48), two important transgenic mouse models corroborate the importance of glucokinase and provide a basis for further study of the glucose-sensing system. The fact that modest overexpression of a yeast hexokinase in transgenic β-cells resulted in augmented glucose-stimulated insulin secretion (49) and protected against β-cell desensitization by low glucose exposure (50) demonstrates that islet hexokinase activity is a critical component of acute glucose sensing and the chronic maintenance of glucose sensitivity. By expressing a glucokinase antisense mRNA sequence with ribozyne activity, Efrat et al. (51) were able to achieve a 70% reduction in islet glucokinase expression, which resulted in a decrease in islet (perfused pancreas) glucose sensitivity. The preservation of normal in vivo glucose homeostasis in these mice suggests that mice may be able to compensate for this degree of impaired glucose sensing, which would be expected to result in the MODY phenotype in affected humans.

Modulation of hepatic glucose production. A relative impairment of insulin's ability to suppress hepatic glucose production is central to the pathogenesis of the syndrome of NIDDM and largely accounts for the increase in fasting glucose concentrations (1). A critical step in hepatic glucose-
TIME (min) INSULIN (nM)

FIG. 3. Impairment of mouse muscle insulin receptor tyrosine kinase activity after transgenic overexpression of a mutant human insulin receptor. A putative dominant-negative human insulin receptor (Ala113→Thr) was overexpressed in the muscle of transgenic mice using the MCK promoter. A: before or 20 and 40 min after in vivo administration of insulin (50 mU/g), gluteal muscle samples were obtained from control (△) or transgenic (△) mice and analyzed for total insulin receptor tyrosine kinase activity. Gluteal muscles from transgenic mice overexpress mutant receptors by 8- to 11-fold, resulting in a marked tyrosine kinase defect. B: isolated soleus muscles, which contain fewer mutant receptors than gluteal muscle, from control (△) versus transgenic (△) mice were assayed for insulin receptor tyrosine kinase activity after in vitro stimulation with increasing insulin concentrations. Data are from Chang et al (18).

ogenesis involves the conversion of oxaloacetate to phosphoenolpyruvate by the enzyme PEPCK. Since PEPCK mRNA transcription is tightly regulated by dietary/hormonal modulation, the PEPCK promoter has been extensively studied. In addition to studies performed in transfected cultured cells (52), PEPCK promoter–bovine growth hormone fusion genes have been introduced into transgenic mice and used to map elements within the promoter that confer liver-specific gene expression and to further characterize a segment containing the cAMP response element (53). A similar transgenic reporter-gene approach has been applied to characterize important regulatory elements within the hepatic (l-type) pyruvate kinase gene promoter, which is regulated in an opposite fashion relative to PEPCK (54).

By introducing multiple copies of a PEPCK minigene into mice, Bosch et al. (55) have succeeded in generating a model with persistently increased PEPCK expression. Interestingly, these mice developed insulin resistance and hyperglycemia, indicating that a primary increase in hepatic glucose production is sufficient to cause a form of NIDDM.

Modulation of insulin sensitivity and action. Numerous defects in insulin signaling or insulin-stimulated glucose uptake/metabolism have been reported using adipocytes and skeletal muscle derived from humans or rodents with NIDDM (56,57). Transgenic methods provide a logical way to address the potential role of any given molecular or cellular alteration by selectively perturbing the function or expression of one specific gene product.

To address the potential role of altered insulin receptor expression or function in insulin resistance and NIDDM, we have used muscle-specific genetic elements (myosin light chain or muscle creatine kinase [MCK]) to drive the expression of normal or mutant human insulin receptors in the muscle of transgenic mice. By achieving a modest (1.7-fold) increase in normal muscle receptor expression, we generated mice with slightly improved in vivo insulin responsiveness (58). These results suggest that modulation of muscle receptor expression could contribute to variations in human insulin sensitivity. Having identified heterozygous insulin receptor missense mutations affecting the receptor tyrosine kinase domain in patients with severe insulin resistance (59), we used the MCK promoter-enhancer to achieve high-level muscle-specific expression of a kinase-deficient human receptor (18). Since these mice were characterized by features of in vivo insulin resistance associated with marked impairment of net muscle insulin receptor kinase activity (Fig. 3), we have verified the dominant-negative hypothesis. Additionally, these results suggest that the modest (acquired) impairment of insulin receptor tyrosine kinase activity that occurs in NIDDM may have physiological consequences, although insulin-stimulated glucose transport was not affected in transgenic soleus muscles, where mutant receptors were expressed at lower levels (18). An additional interesting transgenic model involving the insulin receptor was reported recently. In this model, the soluble insulin receptor ectodomain alone was overexpressed (60). These mice had circulating insulin-binding activity that resulted in chronic mild hyperglycemia; thus, a novel model to study the glucose toxicity hypothesis was created.

A very recent and exciting development concerns targeted disruption of the insulin receptor substrate 1 (IRS-1) gene, which was accomplished simultaneously by two groups (61) (C.R. Kahn, personal communication). Preliminary results obtained with IRS-1 "knock-out" mice reveal that IRS-1 is required for normal growth and development, which results from the mitogenic effects of insulin and insulin-like growth factor 1 (IGF-1). Surprisingly, these mice are not hyperglycemic and display only mildly reduced in vivo insulin sensi-
tivity (for glucose lowering). Thus, an alternative substrate of the insulin receptor kinase that was detected in these mice has been implicated as a potentially important new candidate signaling molecule (C.R. Kahn, personal communication).

Several groups have used transgenic mice to study the effects of altered glucose transporter expression in order to further characterize how modulation of glucose transport might affect in vivo glucose disposal. In addition, transgenic mice have been used to define 5'-flanking regions of the GLUT4 gene that confer expression specific for insulin-responsive tissues (62,63) and result in appropriate regulation by fasting/refeeding (62) or insulin-deficient diabetes (64). Overexpression of the native GLUT4 gene resulted in substantially lower plasma glucose and insulin levels that were associated with a measured increase in basal adipocyte glucose uptake (65). Marked GLUT4 overexpression in transgenic fat was achieved using an adipocyte P2 promoter–GLUT4 fusion gene (66). This manipulation resulted in constitutively increased adipocyte glucose uptake and improved in vivo glucose homeostasis; interestingly, the mice also displayed mild obesity as a consequence of adipocyte hyperplasia (66). Similarly, muscle-specific expression of the GLUT1 transportase isoform resulted in markedly increased basal muscle glucose uptake (67,68). This was associated with an increase in muscle-free glucose and resistance to further stimulation of glucose transport via GLUT4 translocation (69). The following conclusions can be drawn from the experiments cited above: 1) modulation of transporter gene expression can dramatically influence cellular glucose uptake and metabolism; thus, glucose transport is an important rate-limiting step for net glucose disposal, 2) enhanced glucose uptake into muscle and/or fat can improve in vivo glycemia/insulinemia, and 3) altered nutrient partitioning that favors adipocyte glucose uptake may predispose to the development of some forms of obesity.

Approaches to obesity. In addition to the insights into obesity that have been provided by transgenic mice with adipocyte-specific GLUT4 overexpression, the use of standard transgensics or gene-targeting methods to explore the pathogenesis of obesity (or altered body fat distribution) and its relationship to NIDDM is now receiving increased attention. Obesity is the net result of potentially complex physiological derangements that may result in reduced energy expenditure, increased food intake, or both. Transgenic approaches offer a powerful way to perturb specific components, such as candidate satiety factors or molecules implicated in the regulation of thermogenesis. Until very recently, work in this area has been devoted to the use of transgenic mice (and pigs) to study the potential salutary effects of altered nutrient partitioning that favors adipocyte glucose uptake may predispose to the development of some forms of obesity.

Level of the hypothalamus and pituitary. These mice developed obesity as a consequence of reduced energy expenditure that may be related to the increase in peripheral glucocorticoid effect and/or direct effects of the decrease in brain GCR expression (73). Further studies of this mouse model may provide important new insights in the central regulation of in vivo energy expenditure.

Lowell et al. (74) reported a second striking new rodent model of obesity that was generated by ablation of brown adipose tissue (BAT) through the use of a diphtheria toxigenic gene that was expressed using the BAT-specific mitochondrial uncoupling protein (UCP) gene promoter (75). These mice developed morbid obesity, establishing that BAT (and UCP) play a critical role in the maintenance of normal rodent energy balance. Surprisingly, BAT-deficient mice also displayed increased food intake, suggesting that BAT may be required for normal satiety as well as facultative thermogenesis (74). Because BAT-deficient mice developed profound insulin resistance and NIDDM (77) in the absence of elevated glucocorticoid levels (unlike other rodent models), they will serve as an excellent model for further studies of the interrelationship between obesity and NIDDM. Interestingly, the toxigenic ablation approach has also been used to create lipodystrophy by driving the expression of diphtheria toxin in fat (white and brown) using the adipocyte P2 promoter–enhancer (78). The potential role of glycerol-3-phosphate dehydrogenase (GPDH) in energy expenditure via futile cycling was suggested by results obtained with transgenic overexpression of GPDH, a gene normally expressed in BAT and other tissues (76). In this case, mice developed BAT hypertrophy that was associated with reduced amounts of white adipose tissue.

The regulation of thermogenesis and lipolysis by the sympathetic nervous system is thought to be mediated (at least in part) by β3-adrenergic receptors, since these receptors are expressed specifically in brown and white adipose tissue. To explore the potential role of β3-receptors, Suslic et al. (79) have successfully used gene targeting to disrupt the β3-receptor gene in FVB mice. Analysis of the phenotype of these mice is now in progress (B. Lowell, personal communication). Rather than relying on the conventional introduction of correctly targeted ES cells into blastocysts, these investigators identified mice with the disrupted allele after direct microinjection of their vector into mouse zygotes (79). This approach represents an important paradigm for future studies of candidate obesity genes and potentially represents a more expedient way of generating mice with targeted gene mutations.

FUTURE DIRECTIONS AND POTENTIAL STRATEGIES

The experiments reviewed above serve to illustrate the broad range of currently available transgenic strategies that can and will be applied to the pathogenesis of NIDDM and obesity. A groundswell of transgenic reporter gene studies is certain to identify important new cis-acting promoter or enhancer sequences that confer tissue-specific or hormonally regulated expression of genes whose products are critical players in the maintenance of normal glucose homeostasis. With this information in hand, the identification of new transacting factors and signaling pathways that participate in the regulated expression of such genes can then be more rapidly accomplished. Similarly, the role of newly
identified proteins that are implicated in regulation of glucose-stimulated insulin secretion or insulin-mediated cellular signaling can be further explored by 1) the continued use of conventional transgenics to overexpress normal or mutated proteins, 2) improved antisense RNA and transgenic ribozyme strategies that are designed to impair the expression of specific proteins, and 3) the standard use of gene targeting to create knockout mice with null alleles.

A number of recent technical advances have broadened the scope of potential transgenic strategies that can be applied to the pathogenesis of NIDDM. Difficulties in achieving appropriate tissue-specific or regulated expression of transgenes that arise from unfavorable sites of integration may be improved by the use of heterologous matrix-attachment region sequences, which have been recently shown to confer position-independent accurate expression of a whey acidic protein transgene (80). Because germ-line transmission of ES cell-derived alleles is often difficult, new strategies for producing mouse embryos that are completely derived from targeted ES cells are being developed. These methods involve the in vitro aggregation of ES cells (81). Several investigators have devised so-called in-out or hit-and-run gene-targeting schemes that provide a way to introduce subtle changes (e.g., single nucleotide substitutions) into targeted genes. Using ES cells, a selectable marker gene plus the desired mutation is first introduced into the target locus by homologous recombination; the marker sequence is then removed by either spontaneous intrachromosomal recombination (27,82,83) or by the use of a specific recombinase enzyme (84). Alternatively, a second gene-targeting step can be used to replace the marker with the mutant (85). The ability to achieve homologous recombination by direct vector injection (79,86), as discussed above, also remains an attractive prospect that could be applied to introduce subtle genetic defects in future gene-targeting experiments.

Since many standard knockout experiments result in embryonic lethality, the ability to create cell type-specific gene targeting, where the function of a gene product in a given tissue of the living animal can be studied, is a desirable goal. Through the use of a recombinase enzyme (Cre) that cleaves DNA at specific (loxP) sites, a tissue-specific knockout system was recently developed (87,88) and proven to be feasible (89). Advanced gene-targeting strategies that allow the investigator to turn specific genes on or off at will in the adult animal are also forthcoming and will help to distinguish specific alterations in the physiology of glucose and insulin from chronic effects that accrue during development of the organism (26).

It is clear that genetic susceptibility to NIDDM is not inherited in a simple Mendelian fashion, and that with very few exceptions (e.g., glucokinase or insulin receptor mutations), we have just begun to scratch the surface in the search for genetic defects that contribute to the pathogenesis of NIDDM (3). Thus, NIDDM and associated physiological derangements, such as obesity or insulin resistance per se, are complex traits in which (in most cases) a single genetic defect is neither necessary nor sufficient to cause the disorder. Future linkage studies of large NIDDM pedigrees or sib-pair analyses are likely to implicate a number of new genetic loci with major effects. In addition, the use of allelic association (90) to map new quantitative trait loci (QTL) may be a feasible complementary approach. The ability to map QTL for NIDDM-associated traits using recombinant inbred strains of mice will prove to be a rapid way in which new candidate diabetes genes for human NIDDM can be identified (91). The analysis of specific new candidate genes, which become known through nongenetic studies, is also likely to yield a large number of potentially important sequence variants that affect promoter regions or the predicted protein gene products.

Transgenic animals provide powerful tools that can be used to assess the potential contributions of candidate loci, candidate genes, or candidate mutations to NIDDM-related phenotypes. Since different transgenes or targeted alleles can be readily combined by interbreeding (92), animal models with one or more genes that bear specific mutations identified in humans can be generated, studied, and treated with new drugs that are under development. The analysis of candidate loci, which are identified by positional cloning in mice or humans, will prove to be a daunting task. Since large regions of genomic DNA can be isolated using yeast artificial chromosomes (YACs) and mice that bear and express full-length YAC transgenes can be generated (93,94), YAC transgenics may soon be used to introduce or rescue potentially defective loci well before specific genes or mutations have been identified or exhaustively analyzed.

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