Mutations in Insulin-Receptor Gene in Insulin-Resistant Patients

Simeon I. Taylor, MD, PhD
Takashi Kadowaki, MD
Hiroko Kadowaki, MD
Domenico Accili, MD
Alessandro Cama, MD
Catherine McKeon, PhD

Defects in insulin-receptor function have been associated with insulin-resistant states such as obesity and non-insulin-dependent diabetes mellitus (NIDDM). Several types of mutations in the insulin-receptor gene have been identified in patients with genetic syndromes of extreme insulin resistance. In some patients, insulin resistance results from a decrease in the number of insulin receptors on the cell surface. In one patient with leprechaunism (leprechaun/Minn-1), there is >90% decrease in the levels of insulin-receptor mRNA. This patient is a compound heterozygote for two mutations in the insulin-receptor gene, both of which act in a *cis*-dominant fashion to decrease levels of mRNA transcribed from that allele. In one allele, there is a nonsense mutation at codon 897. All 22 exons of the other allele have a normal sequence, so that the mutation in this allele appears to map outside the coding sequence of the gene. Impaired insertion in the plasma membrane also causes insulin resistance. In two sisters (patients A-5 and A-8) with type A extreme insulin resistance, there is an 80–90% decrease in the number of insulin receptors expressed on the surface of their cells. Both sisters, whose parents are first cousins, are homozygous for a point mutation in which valine is substituted for phenylalanine at position 382 in the α-subunit of the insulin receptor. This mutation retards the posttranslational processing of the receptor and impairs the transport of receptors to the cell surface. Another patient with leprechaunism (leprechaun/Ark-1) is a compound heterozygote with two different mutant alleles of the insulin-receptor gene. In the allele derived from the father, there is a nonsense mutation at codon 672 that truncates the insulin receptor by deleting the COOH-terminal of the α-subunit and the entire β-subunit. This truncated receptor, lacking a transmembrane domain, appears not to be expressed at the plasma membrane. In leprechaun/Ark-1, there is a missense mutation in the allele of the insulin-receptor gene derived from the mother. This point mutation results in substitution of glutamic acid for lysine at position 460 in the COOH-terminal half of the α-subunit. This mutation increases receptor affinity and impairs the ability of acid pH to dissociate insulin from the receptor within the endosome. There is a defect in recycling the receptor back to the plasma membrane associated with this defect. This results in an accelerated rate of receptor degradation and a consequent decrease in the number of receptors on the cell surface in vivo. Some patients have a normal number of receptors on their cell surface, but there is a defect in receptor function. A mutation has been described in the tetrabasic amino acid sequence separating the α- from the β-subunit. This mutation, identified in two sisters from a consanguineous pedigree, inhibits the cleavage of the receptor precursor into separate subunits. The uncleaved receptor has a decreased affinity to bind insulin. An insulin-resistant patient has been identified in whose insulin receptors there is a defect in tyrosine kinase activity due to a mutation that substitutes valine for glycine-1008, the third glycine in the conserved Gly-X-Gly-X-X-Gly motif in the ATP-binding domain of the β-subunit of the insulin receptor. The patient is heterozygous for this mutation. All of the mutations in the insulin-receptor gene that have been identified so far have been detected in patients with rare clinical syndromes associated with extreme insulin resistance. Recent advances in recombinant DNA technology have greatly simplified the task of detecting mutations. Thus, it should soon be possible to determine the prevalence of mutations in the insulin-receptor gene. These studies will also answer the question of whether mutations in the insulin-receptor gene contribute to the pathogenesis

From the Biochemistry and Molecular Pathophysiology Section, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland.

Address correspondence and reprint requests to Dr. Simeon I. Taylor, National Institutes of Health, Building 10, Room 8N-250, Bethesda, MD 20892.

of insulin resistance with common forms of NIDDM.
***Diabetes Care* 13:257–79, 1990**

Insulin resistance plays a central role in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM). Furthermore, the predisposition to develop NIDDM appears to be genetically determined. Thus, it is of interest to determine whether there are genetic factors that cause insulin resistance. Because of the key function of the insulin receptor in mediating the first step in insulin action, a mutation in the insulin-receptor gene is a plausible candidate to account for genetic forms of insulin resistance. Over the past two decades, many patients have been identified with genetic diseases associated with insulin resistance and defects in insulin-receptor function (1–3). Since the cloning of insulin-receptor cDNA in 1985 (4,5), it has been possible to confirm the hypothesis that many of these patients have mutations in their insulin-receptor gene (6–13). In this review, we summarize current understanding of the molecular pathogenesis of genetic syndromes associated with insulin resistance. Recent advances in recombinant DNA technology have simplified the task of determining the nucleotide sequence of genes. This will greatly facilitate future studies to determine whether mutations in the insulin-receptor gene contribute to the pathogenesis of insulin resistance in the common forms of NIDDM.

GENETIC SYNDROMES OF EXTREME INSULIN RESISTANCE

From the point of view of the clinical investigator, patients with inborn errors in the pathways of insulin action represent intriguing experiments of nature (1–3). The severity of the insulin resistance has greatly facilitated studies into the biochemical and molecular mechanisms of insulin resistance. Although these patients manifest an extreme degree of resistance to the biological actions of insulin, many of the patients are not diabetic. In some patients, the levels of insulin rise to the point at which they are sufficiently high (often 10- to 100-fold above the normal range) to maintain normal glucose tolerance. Nevertheless, some patients, especially patients with the most severe degree of insulin resistance, develop fasting hyperglycemia and overt diabetes. At least two mechanisms contribute to the development of hyperinsulinemia in insulin-resistant patients. First, as reflected by the increase in the levels of C-peptide in plasma, the β-cell increases the rate of insulin secretion. Second, because receptor-mediated endocytosis is the principal route by which insulin is cleared from plasma, a decrease in the number of insulin receptors on the cell surface decreases the insulin clearance rate (14).

Two clinical features are commonly observed in patients with all of the syndromes of extreme insulin resistance, regardless of the biochemical mechanism that causes the insulin resistance:

1. Acanthosis nigricans. This is a hyperkeratotic hyperpigmented skin lesion located primarily in skin folds such as the axillae and antecubital fossae. It tends to correlate with hyperinsulinemia. In patients in whom insulin resistance is caused by anti-receptor autoantibodies, the acanthosis nigricans waxes and wanes in association with the appearance and disappearance of the insulin resistance. These observations have led to the hypothesis that acanthosis nigricans may be caused by a toxic effect of hyperinsulinemia on the skin (1–3).
2. Hyperandrogenism. Levels of plasma testosterone are commonly elevated in premenopausal women with extreme insulin resistance (1–3,15,16). Elevated levels of testosterone result from overproduction of testosterone by the ovaries. As with acanthosis nigricans, elevated levels of testosterone correlate with hyperinsulinemia. Clinically, the elevated levels of testosterone are manifested as a syndrome of polycystic ovaries, oligomenorrhea, and hirsutism.

Although all of the syndromes of extreme insulin resistance share some features in common, multiple distinct syndromes can be defined based on the presence or absence of specific clinical features. For example, type A extreme insulin resistance is defined by the triad of insulin resistance, acanthosis nigricans, and hyperandrogenism in the absence of obesity or lipoatrophy (1). In lipoatrophic diabetes, there is atrophy of subcutaneous fat, hypertriglyceridemia, and fatty metamorphosis of liver (17). Patients with leprechaunism have multiple abnormal features, including intrauterine growth retardation and fasting hypoglycemia (18). Rabson-Mendenhall syndrome is associated with abnormalities of the teeth and nails and, reportedly, pineal hyperplasia (19,20).

INSULIN RECEPTORS

HUMAN INSULIN-RECEPTOR GENE

The gene encoding the human insulin receptor is composed of 22 exons and spans in excess of 120 kilobase (kb) on chromosome 19 (21,22). There are multiple start sites for transcription in the region 300–600 base pair upstream from the initiator methionine codon (23–25). Five species of mRNA ranging in size from 5 to 11 kb have been identified (4,26; Fig. 1). The major variation in size is due to variable lengths of 3′-untranslated RNA, presumably resulting from the use of alternate polyadenylation signals (25,27). At least one example of alternate splicing has been identified. Exon 11, encoding amino acids 720–731, can either be included or excluded from mature mRNA (28,29). In some tissues, such as the liver, most receptor mRNA contains the nucleotide sequence corresponding to exon 11. Other cell

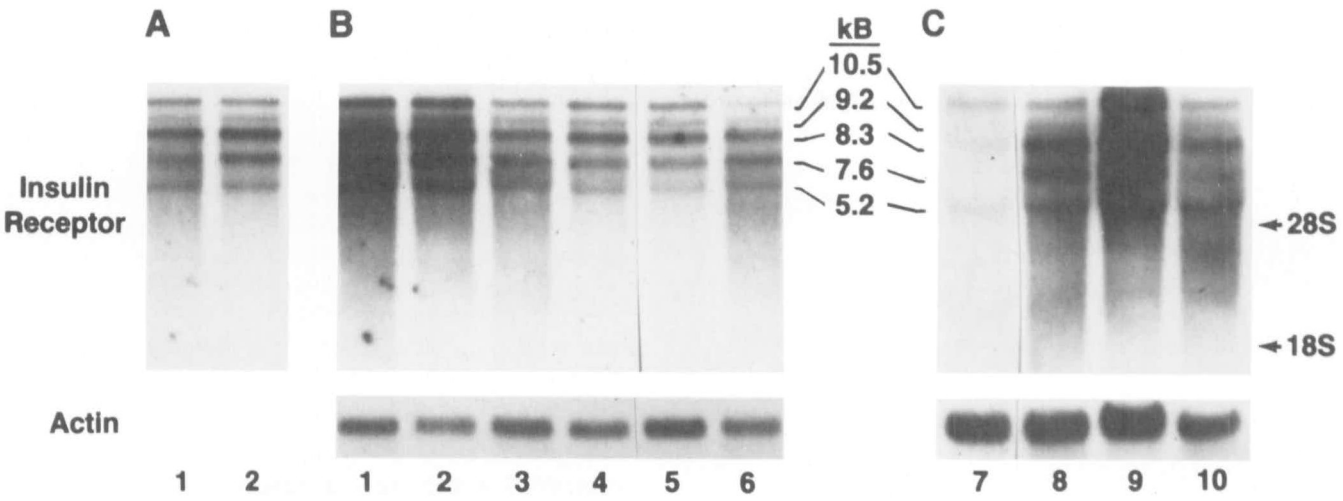

FIG. 1. Northern blot analysis of insulin-receptor mRNA levels in cells from patients with decreased numbers of insulin receptors on cell surface. Polyadenylated RNA from Epstein-Barr virus–transformed lymphoblasts was analyzed by Northern blotting. Blots were probed with either human insulin-receptor (*upper panels*) or chicken β-actin (*lower panels*) probes (26). *A*: autoradiograph of *lanes 1* and *2* with exposure time reduced from 3 to 1 day to allow for better visualization of insulin-receptor mRNA bands. *B*: cells from patients A-5 and A-8 (*lanes 3* and *4*) had normal levels of insulin-receptor mRNA. Blots of mRNA from healthy individuals are shown in *lanes 1, 2, 5,* and *6*. *C*: reduction in insulin-receptor mRNA in leprechaun/Minn-1 (*lane 7*) compared with healthy subjects (*lanes 8–10*).

types (e.g., cultured Epstein-Barr virus [EBV]–transformed lymphoblasts) primarily contain the form lacking the exon 11 sequence. However, many tissues (e.g., placenta) contain both splicing variants of insulin-receptor mRNA. It has been reported that deletion of the 12 amino acids encoded by exon 11 increases the affinity to bind insulin (30), although another laboratory failed to confirm this observation (P. De Meyts, unpublished observations).

Structure of insulin receptor. The insulin-receptor gene encodes a single polypeptide that undergoes *N*-linked glycosylation to yield a 190,000-*M*, precursor of the insulin receptor (4,5,31–34). This undergoes additional posttranslational processing to yield the mature receptor (Table 1). First, the precursor undergoes proteolytic cleavage into two separate subunits (32–34). Second, the high-mannose form of *N*-linked carbohydrate undergoes maturation with removal of mannose and glucose residues and addition of other sugars including sialic acid (32). Other posttranslational modifications occur, including fatty acylation and *O*-linked glycosylation (35–37). Thus, the basic structural unit of the mature insulin receptor is a heterodimer formed by α- and β-subunits. The α- and β-dimers further dimerize to produce a heterotetrameric $\alpha_2\beta_2$ species (Fig. 2).

TABLE 1
Posttranslational modifications of insulin receptor

Proteolytic cleavage into subunits
Glycosylation
N-linked (α- and β-subunits)
O-linked (β-subunit)
Fatty acylation
Amide (α- and β-subunits)
Ester (β-subunit)
Phosphorylation
Tyrosine
Serine/threonine

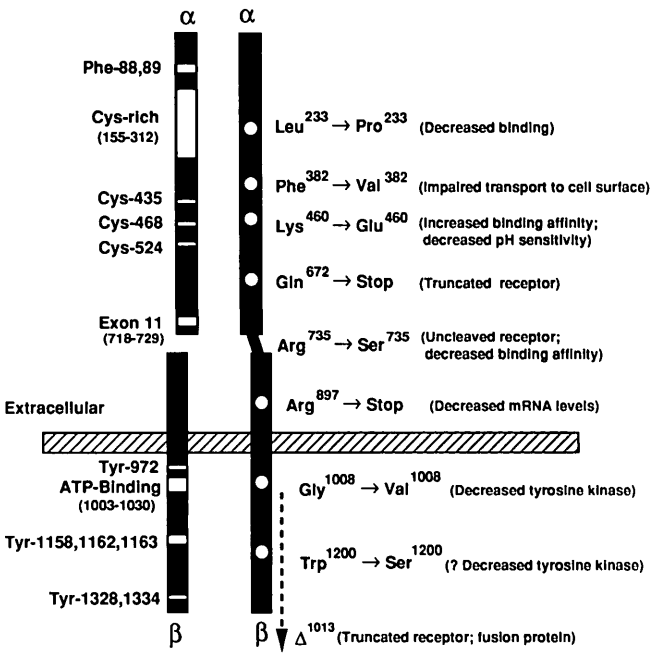

FIG. 2. Mutations in insulin-receptor gene in insulin-resistant healthy patients. Structural map of insulin receptor. Key structural landmarks are identified on *left*. Locations of all of mutations reported to cause insulin resistance are noted on *right* (6–13,65).

α-Subunit. The α-subunit is extracellular and provides the binding site for insulin (23,38,39). The binding site has not been definitively mapped. However, two lines of evidence suggest that the binding site may be located in the NH_2-terminal portion of the α-subunit. First, when the receptor is affinity labeled with ^{125}I-labeled insulin, a point of covalent attachment has been mapped to the NH_2-terminal ~300 amino acids of the α-subunit (40, 40a). Second, substitution of leucine for Phe^{88} or Phe^{89} by site-directed mutagenesis decreases the receptor's binding affinity (41).

Nevertheless, when a deleted cDNA encoding only the α-subunits was expressed by transfection into 3T3 cells, it was not possible to detect a truncated receptor with high binding activity, possibly because isolated α-subunits are unstable (42). In contrast, similar transfection studies have demonstrated that a truncated receptor containing the α-subunit plus the extracellular domain of the β-subunit does retain insulin-binding activity. This suggests that the receptor's β-subunit may participate directly or indirectly in forming the insulin-binding site. This conclusion is further supported by the observation that it is possible to affinity label the β-subunit with ^{125}I-insulin, albeit with 10-fold lower efficiency than the α-subunit.

When a bivalent cross-linking reagent such as disuccinimidyl suberate is added to the tetrameric receptor, it is possible to covalently cross-link two α-subunits. Furthermore, in the native receptor, there appear to be disulfide bonds between adjacent α-subunits that have been tentatively mapped to the cysteine residues at positions 435, 468, and/or 524 (43).

β-Subunit. The β-subunit contains the transmembrane domain that anchors the receptor in the plasma membrane and possesses enzymatic activity as a tyrosine-specific protein kinase (44–46; Fig. 2). When insulin binds to the extracellular domain of the receptor, the receptor tyrosine kinase activity is activated. Evidence supports the hypothesis that activation of tyrosine kinase plays a necessary role in mediating insulin action on the target cell (46). Site-directed mutagenesis of the insulin receptor provides some of the most convincing evidence.

The site for binding of the substrate ATP is highly conserved in all protein kinases. The conserved ATP-binding motif in the insulin receptor consists of the Gly^{1003}-X-Gly^{1005}-X-X-Gly^{1008}....-Lys^{1030} sequence (47). Mutation of Lys^{1030} abolishes the tyrosine kinase activity of the receptor. Furthermore, it abolishes the ability of the receptor to mediate the various biological actions of insulin (48,49). In addition, another mutation in the ATP binding site, substitution of valine for Gly^{1008}, impairs tyrosine kinase activity and causes insulin resistance in vivo (8).

Autophosphorylation of tyrosine residues in the receptor results when insulin binds to the receptor (44–46). Receptor autophosphorylation stimulates activity of tyrosine kinase to phosphorylate other protein substrates (46,50–53). There are at least two clusters of tyrosine residues that are autophosphorylated in response to insulin: tyrosine residues 1158, 1162, and 1163 and tyrosine residues 1328 and 1334 (54,55). When Tyr^{1162} and Tyr^{1163} are replaced with phenylalanine, tyrosine kinase activity and the ability of the receptor to mediate insulin action are impaired (56). In addition, another potential phosphorylation site is located at Tyr^{972}, adjacent to an acidic amino acid (Glu^{971}) in a consensus sequence for a tyrosine phosphorylation site. However, there is disagreement in the literature regarding whether Tyr^{972} is phosphorylated (54,55). When Tyr^{972} is mutated to phenylalanine, the ability of the receptor to mediate insulin action is impaired, possibly by impairing phosphorylation of substrates by tyrosine kinase (57).

MOLECULAR MECHANISMS OF INSULIN RESISTANCE

The mechanism of insulin action involves multiple steps, many of which have not yet been characterized in detail. Defects in any of these steps may give rise to insulin resistance. Inasmuch as the insulin receptor is responsible for mediating the first step in insulin action, it is appropriate that investigations of the mechanisms of insulin resistance have begun with studies of the insulin receptor (2,3). Over the past several years, there has been considerable progress in identifying mutations in the insulin-receptor gene in patients with genetic forms of insulin resistance. In theory, mutations might occur in either the domain of the gene encoding the structure of the insulin receptor or in the regulatory domains that determine the level of gene expression. However, all of the reported mutations have been identified in the structural gene (Fig. 2). These mutations impair insulin action by several mechanisms (Table 2).

DECREASED NUMBER OF INSULIN RECEPTORS ON CELL SURFACE

In some patients, insulin resistance results from a decrease in the number of insulin receptors on the cell surface. This type of defect has been observed in patients with type A extreme insulin resistance (58,59),

TABLE 2
Molecular mechanisms of insulin resistance

Decreased number of insulin receptors on surface of target cells
Decreased levels of insulin-receptor mRNA
Mutations in regulatory regions of gene
Mutations at intron-exon boundaries that impair RNA splicing
Nonsense mutations (most but not all)
Mutations that accelerate rate of mRNA degradation
Mutations in genes encoding *trans*-acting transcription factors
Impaired transport of receptors to cell surface
Nonsense mutation
Accelerated receptor degradation (impaired receptor recycling)
Defects in insulin-receptor function
Decreased affinity of receptor to bind insulin
Decreased receptor-associated tyrosine kinase activity

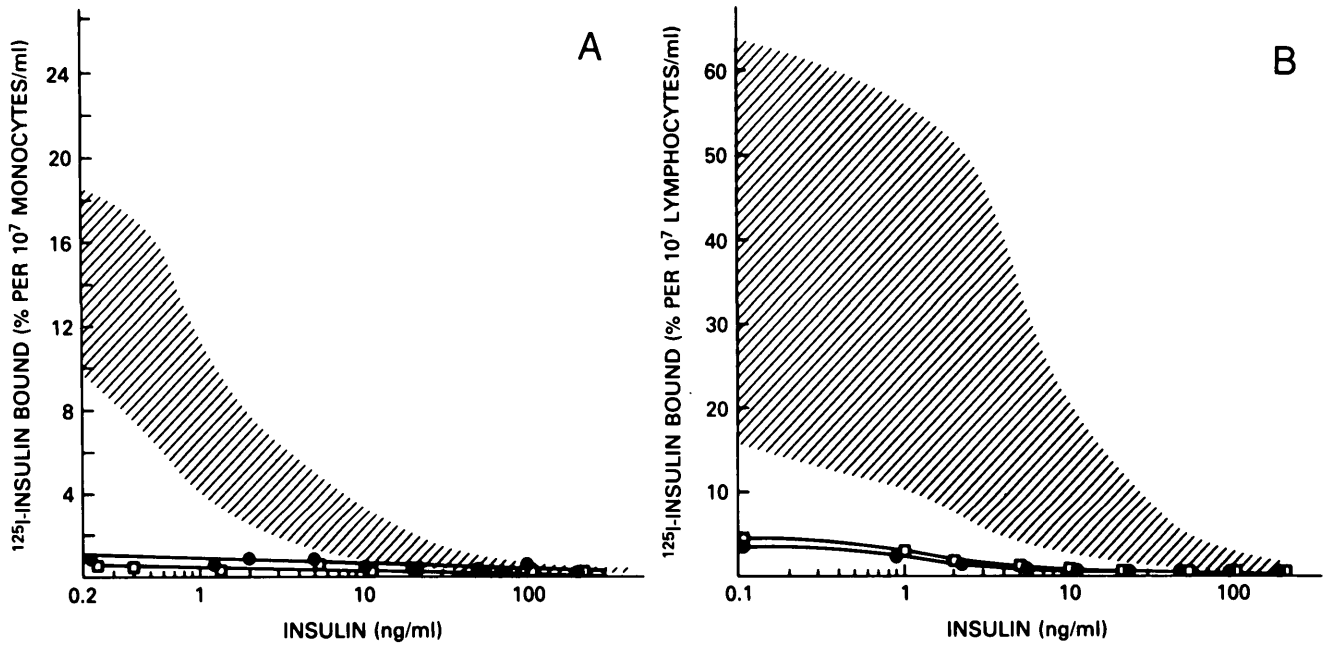

FIG. 3. Decreased insulin binding to surface of cells from 2 sisters (patients A-5 [●] and patient A-8 [□] with type A extreme insulin resistance. Circulating monocytes (*A*) and Epstein-Barr virus–transformed lymphoblasts (*B*) were incubated with ^{125}I-insulin (0.1 ng/ml) plus varying concentrations of unlabeled insulin. Percentage of ^{125}I-insulin bound specifically is plotted as a function of insulin concentration. *Cross hatched area* is range of binding observed in healthy subjects (3).

leprechaunism (58), and Rabson-Mendenhall syndrome (60). The decrease in receptor number was first detected in circulating monocytes and erythrocytes from patients with type A extreme insulin resistance. Such a defect has also been confirmed in subcutaneous adipocytes, a classic target cell for insulin action (61). With these cell types, studies were conducted immediately after cells were obtained from the patient. However, the defect is preserved in lymphoblastoid cells that have been immortalized by transformation with EBV (58,60; Fig. 3). Because EBV-transformed lymphoblasts are cultivated in vitro under standard conditions in the absence of insulin, the decrease in the number of insulin receptors may be caused by a primary defect intrinsic to the cell. Of course, the elevated levels of insulin in the plasma of these insulin-resistant patients may cause downregulation, thereby exacerbating the decrease in the number of insulin receptors in target cells in vivo.

Despite the common biochemical phenotype observed in this group of patients, at least four different molecular defects have been responsible for the decrease in the number of insulin receptors on the surface of patients' cells: *1*) decreased levels of insulin-receptor mRNA, *2*) impaired transport of insulin receptors to the cell surface, *3*) nonsense mutations, and *4*) accelerated receptor degradation.

Decreased levels of insulin-receptor mRNA. In studies of EBV-transformed lymphoblasts from leprechaun/Minn-1, we demonstrated that the number of insulin receptors on the cell surface is decreased to <10% of normal (58). Furthermore, as judged by the rate at which radioactively labeled amino acids and sugars were incorporated into the receptor precursor, the rate of receptor biosynthesis is decreased sufficiently to account for the decrease in the number of insulin receptors (Fig. 4). A similar decrease in the rate of receptor biosynthesis has been observed in a patient with type A extreme insulin resistance (patient A-1; 62).

Because insulin-receptor mRNA is necessary to provide a template for receptor biosynthesis, we measured the level of insulin-receptor mRNA in cells from leprechaun/Minn-1. Although all five species of human insulin-receptor mRNA are present in the patient's cells, there was a marked decrease in the cellular content of insulin-receptor mRNA (26; Fig. 1C). Recent studies confirmed that there is a decrease in the level of insulin-receptor mRNA (63) and the number of cell surface insulin receptors (64) in cultured fibroblasts from this patient. A mutation has been identified in the allele of the insulin-receptor gene inherited from her father: a nonsense mutation at codon 897 in exon 14 (65). As with many nonsense mutations in other genes, this nonsense mutation is associated with a reduction in the level of mRNA transcribed from that allele. Although we determined the nucleotide sequence of the protein-coding regions of all 22 exons together with the intron-exon junctions of both alleles, we have not identified a mutation in the maternal allele. Nevertheless, indirect evidence strongly suggests that there is a *cis*-dominant mutation in the maternal allele (65). Studies are underway

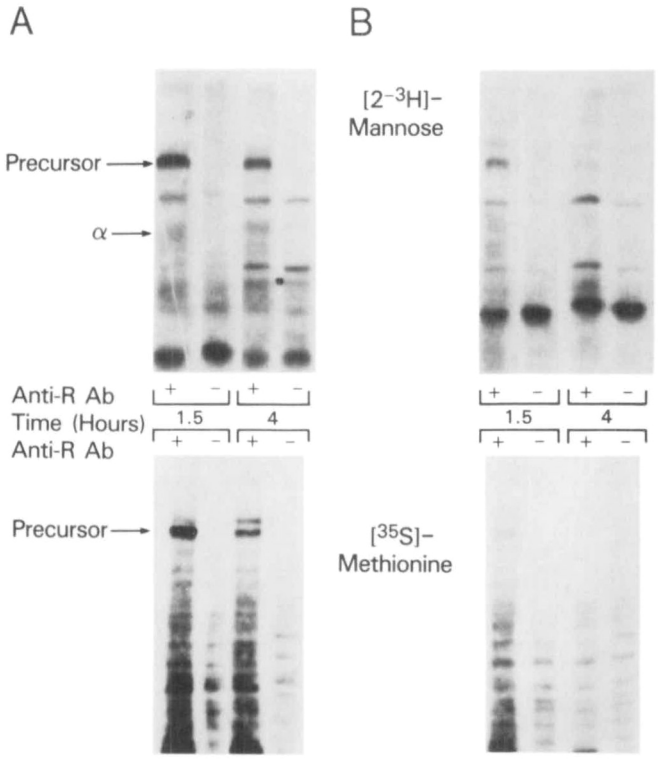

FIG. 4. Decrease in rate of receptor biosynthesis in cells from leprechaun/Minn-1. Epstein-Barr virus–transformed lymphoblasts from healthy subject (*A*) and leprechaun/Minn-1 (*B*) were pulse-labeled with [2-^{3}H]mannose (*upper panels*) or [^{35}S]methionine (*lower panels*). After chase period of 1.5 or 4 h, receptors were solubilized and immunoprecipitated and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. +, Immunoprecipitation with anti-receptor antiserum; −, immunoprecipitation with nonimmune human serum (26).

to determine whether this mutation decreases the level of insulin-receptor mRNA by decreasing the rate of synthesis or accelerating the rate of degradation of the mRNA encoded by the maternal allele of the insulin-receptor gene. Leprechaun/Minn-1 appears to be a compound heterozygote for two different *cis*-dominant mutations in the insulin-receptor gene. Although it has not been possible to investigate the patient's father, her mother has a 50% decrease in insulin binding to the surface of her circulating monocytes. Thus, the decrease in insulin binding is inherited in a codominant fashion.

Impaired transport of insulin receptors to cell surface. We studied two sisters with type A extreme insulin resistance (patients A-5 and A-8) in whom there was an 80–90% decrease in the number of insulin receptors on the cell surface (12; Fig. 3). However, unlike what was observed in leprechaun/Minn-1, we detected normal levels of insulin-receptor mRNA in EBV-transformed lymphoblasts from patients A-5 and A-8 (26; Fig. 1*B*). Moreover, the rate of receptor biosynthesis was normal (62). Why then was the number of receptors on the cell surface decreased? This does not result from an accelerated rate of receptor degradation. When receptors on the surface of EBV-transformed lymphoblasts from patient A-5 were labeled by lactoperoxidase-catalyzed radioiodination and the rate of receptor degradation was measured, the half-life of the patient's receptors was within normal limits ($t_{1/2}$ = 4.8 h, normal range 6.5 ± 2.4 h, mean ± 2SD) (66). This led to the hypothesis that there is a defect in the transport of insulin receptors to the plasma membrane. We described a similar biochemical phenotype in an insulin-resistant patient with Rabson-Mendenhall syndrome (67).

A defect in the transport of receptors to the cell surface has also been described with low-density lipoprotein (LDL) receptors in the Watanabe heritable hyperlipidemic rabbit and some patients with familial hypercholesterolemia (68,69). In these cases, mutations were detected in the structural gene encoding the LDL receptor. The mutant LDL receptors were impaired in their ability to be transported to the cell surface. Based on this analogy, we cloned insulin-receptor cDNA from patient A-8 and identified a single amino acid substitution in the patient's insulin receptor: substitution of valine for phenylalanine at position 382 in the α-subunit (12; Fig. 2). Two independent lines of evidence support the conclusion that this mutation is the cause of the patients' disease: genetic linkage and expression of the mutant insulin receptor by transfection.

The pedigree of Val^{382} is shown in Fig. 5. The mode of inheritance of the disease in this kindred is certainly autosomal recessive, inasmuch as the parents are first cousins and the phenotype was not observed in earlier generations. In addition to the two insulin-resistant sisters (patients A-5 and A-8), there are four unaffected siblings. According to this model of inheritance, three predictions can be made: *1*) the two parents are heterozygous carriers of the mutant allele inherited by descent from a common great-grandparent who was a heterozygous carrier of the mutation, *2*) the two patients

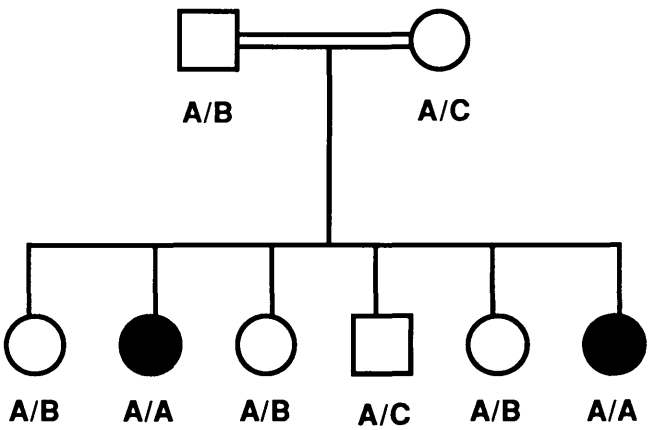

FIG. 5. Pedigree of consanguineous kindred of 2 insulin-resistant sisters (A-5 and A-8). Parents of patients A-5 and A-8 are first cousins (12,65). ●, Affected sisters; ○, unaffected family members. Allele A is allele with Phe^{382} mutation. Alleles B and C are normal alleles observed in father and mother, respectively.

are homozygous for the same mutant allele, and 3) each of the four unaffected siblings is either a heterozygous carrier of the mutation or homozygous normal.

When the Val^{382} mutation was used as a marker for the mutant allele, this was the same inheritance pattern we observed in the kindred (Fig. 6). Because this pattern of inheritance is unlikely to have occurred as the result of random chance (probability ~1/200), this provides statistical evidence that Val^{382} is genetically linked to the disease-causing mutation (12,70).

Strictly speaking, this analysis is oversimplified. Because we have not studied the grandparents and great-grandparents, we are not certain that the patients are truly homozygous for an allele that is identical by descent from a single great-grandparent. It is possible that the two alleles encoding valine at position 382 could have been inherited from two different great-grandparents. Thus, statistical analysis must be adjusted for the degree of confidence that the two mutant alleles are identical by descent. Lander and Botstein (70) introduced a new technique called homozygosity mapping to calculate $\log_{10}$ of odds ratio (LOD) scores for genetic linkage in consanguineous pedigrees. We applied this technique to study this pedigree (12). Obviously, if the Val^{382}-containing allele is a frequent allele in the population, then the parents could have inherited the same allele by random chance rather than by descent from the common grandparent. On the other hand, if the Val^{382}-containing allele is rare in the population, then it is likely that the parents have inherited this allele by descent from their common ancestor. We obtained data that strongly supports the conclusion that the allele is rare. Therefore, it is likely that both parents inherited the mutant allele by descent from the same great-grandparent.

We used allele-specific oligonucleotide hybridization to search for the Val^{382} mutation in healthy individuals, but did not detect this mutation in any of the 160 alleles of the insulin-receptor gene in 80 healthy individuals (Fig. 6). Thus, this mutation is rare in the general population (12).

We investigated restriction-fragment–length polymorphisms (RFLPs) of the insulin-receptor gene. As expected, if the alleles were identical by descent, none of the seven RFLPs differentiated the Val^{382}-containing allele observed in the mother from the Val^{382}-containing allele observed in the father (12).

These studies support the conclusion that the two sisters are homozygous for an extremely rare allele of the insulin-receptor gene. Thus, the patients inherited both copies of the mutant allele from the same great-grandparent (i.e., homozygosity by descent). Thus, the LOD score in favor of linkage of the disease-causing mutation to the insulin-receptor gene was ~2.2–2.3 (12; Fig. 7).

To evaluate the significance of the substitution of valine for phenylalanine at position 382, the mutant form of the insulin-receptor cDNA was expressed by transfection in cultured cells. This demonstrated that the Val^{382} mutation impairs posttranslational processing of the receptor precursor and decreases the number of receptors on the cell surface (12).

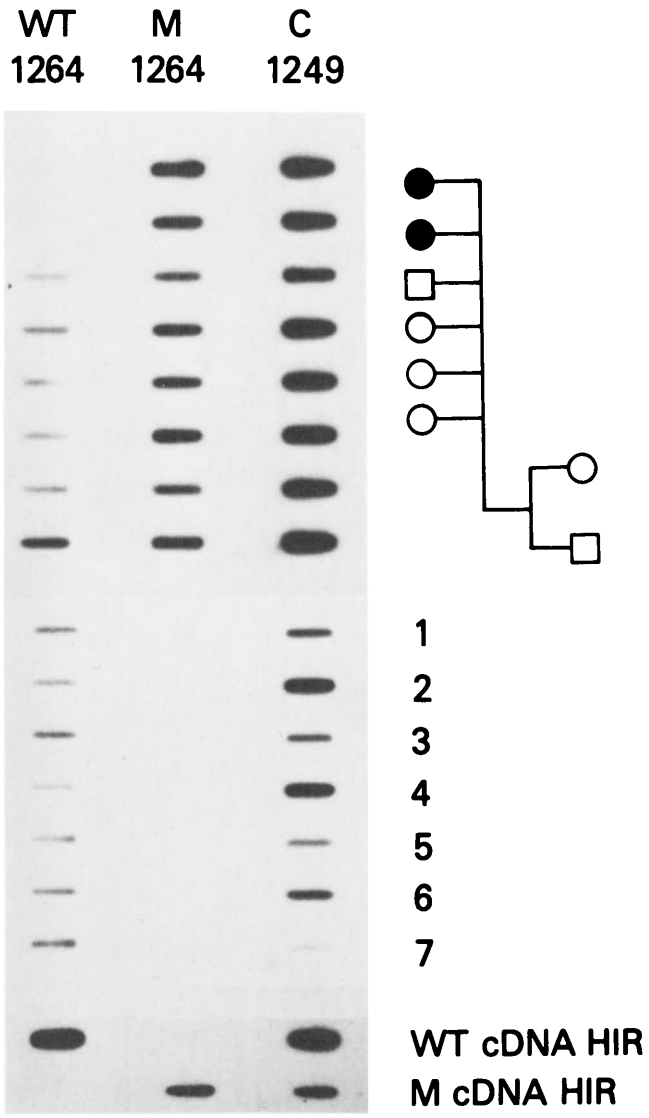

FIG. 6. Inheritance of Val^{382} mutation as detected by allele-specific oligonucleotide hybridization of amplified genomic DNA. One hundred thirty-four base pair fragment of genomic DNA corresponding to nucleotides 1172–1306 of insulin-receptor cDNA was amplified with *Taq* DNA polymerase (12). Thereafter, amplified DNA was applied to nitrocellulose filter with slot-blot apparatus. Blot was hybridized to sequence specific oligonucleotides corresponding to nucleotides 1264–1281 of either wild-type sequence (WT 1264), mutant sequence (M 1264), or control sequence (nucleotides 1249–1266) located upstream from mutation (C 1249). *Symbols* indicate amplified genomic DNA from family members in *upper 8 rows*. *Rows 1–7* indicate amplified DNA from 7 representative healthy individuals. Seventy-three additional healthy individuals were studied (not shown). As methodological control, hybridization to clones containing either wild-type (WT) or mutant (M) sequences is shown in *bottom 2 rows*.

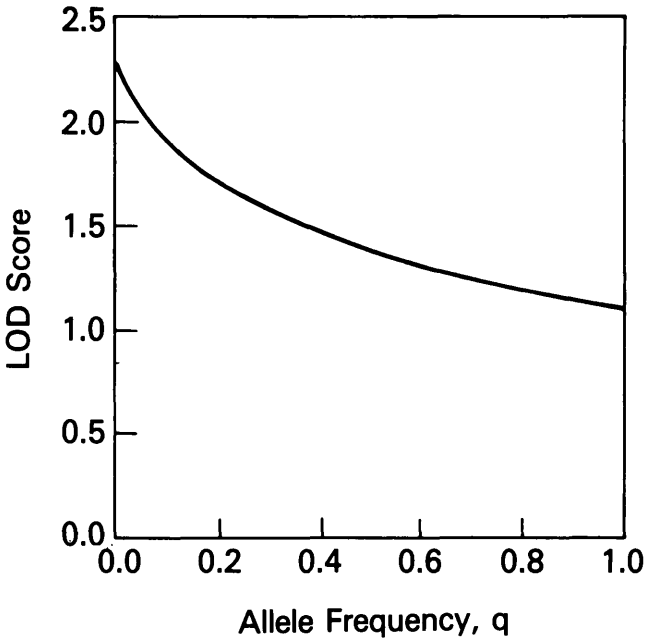

FIG. 7. Dependence of $\log_{10}$ of odds ratio (LOD) score on probability of homozygosity. Odds ratio is defined as ratio of probability of observations assuming that 2 loci are genetically linked divided by the probability of same observations assuming that loci are unlinked. According to method of homozygosity mapping, LOD score in consanguineous family depends on frequency of haplotype for which affected individuals are homozygous. For first-cousin marriage, rare disease, and tightly linked restriction-fragment–length polymorphisms, LOD score is increased by $\log_{10}$ $16/(1 + 15q)$ where q is frequency of observed homozygous haplotype. Details of this calculation have been published elsewhere (12).

To evaluate posttranslational processing of the 190,000 M_r precursor to the mature receptor, we conducted experiments with NIH/3T3 cells transfected with cDNA encoding either normal insulin receptors (WT-8) or insulin receptors with the Val^{382} mutation (V382-2). The transfected cells were pulse labeled with [^{35}S]methionine for 3 h followed by a chase period of 1.5 or 4 h (Fig. 8). These pulse-chase studies provide evidence for a defect in the posttranslational processing of the mutant receptor. In the cells transfected with wild-type cDNA, two bands (M_r 130,000 and 100,000) were seen in the regions of the α- and β-subunits. In the cells transfected with mutant cDNA, two bands (M_r 120,000 and 130,000) were seen in the region of the α-subunit and two bands (M_r 900) in the region of the β-subunit. Previous studies had suggested that proteolytic cleavage of the receptor precedes maturation of the *N*-linked carbohydrate moieties (32,71). Therefore, it seemed likely that the low-molecular-weight forms of the α- and β-subunits represent precursors in which the *N*-linked oligosaccharides had not undergone final processing in the golgi. As suggested previously, the kinetics of labeling are consistent with the 90,000- and 120,000-M_r bands being precursors of the 100,000- and 130,000-M_r bands, respectively. Specifically, the ratio of mature α- and β-subunits (M_r 130,000) to the lower-molecular-weight precursor forms (M_r 120,000) increases as a function of time during the chase period. Thus, posttranslational processing of the 190,000 M_r proreceptor is less efficient in the cells transfected with mutant cDNA.

Is the defect in posttranslational processing associated with impaired transport of the mutant receptor to the cell surface? To address this question, three transfected cell lines that expressed similar levels of insulin-receptor mRNA were studied: two cell lines expressing normal insulin-receptor cDNA (WT-2 and WT-8) and one cell line expressing Val^{382} mutant insulin-receptor cDNA (V382-1). As demonstrated by Scatchard plots of [^{125}I]iodoinsulin binding, cells transfected with wild-type insulin-receptor cDNA (WT-2 and WT-8) express ~5- to 10-fold more insulin receptors on their cell surface than cells transfected with Val^{382} mutant insulin-receptor cDNA (V382-1) (12; Fig. 9). We also studied a cell line (V382-2) transfected with Val^{382} mutant insulin-receptor cDNA that expressed 10-fold higher levels of insulin-receptor mRNA. These studies showed that this 10-fold overexpression of mutant insulin-receptor mRNA allowed for expression of as many insulin receptors on the surface of V382-2 cells as were observed on WT-2 and WT-8 cells.

Nonsense mutation. We studied another patient with leprechaunism (leprechaun/Ark-1) in whom we identified two mutant alleles of the insulin-receptor gene (6,72–75; Fig. 2). In the allele inherited from the father, there was a nonsense mutation substituting the amber chain termination codon (UAG) for the CAG codon normally encoding Gln^{672}. Frequently, nonsense mutations are associated with decreased levels of mRNA (65,76–78). However, this nonsense mutation was associated with normal levels of mRNA. In fact, approximately half of the clones in the cDNA library were derived from the allele of the insulin-receptor gene with the nonsense mutation. This premature chain termination leads to deletion of the COOH-terminal of the α-subunit and the entire β-subunit, including the transmembrane anchor and the tyrosine kinase domain. Lacking a transmembrane anchor, the truncated receptor is not located at the cell surface (73). In fact, the truncated receptors have not been identified in the patient's EBV-transformed lymphoblastoid cells; neither have the truncated receptors been identified as secretory products in the culture medium. Accordingly, it is likely that the truncated receptor is rapidly degraded.

In the patient (leprechaun/Ark-1), the allele of the insulin-receptor gene with the nonsense mutation is paired with an allele containing a different mutation (6). However, the patient's father is heterozygous for the allele with the nonsense mutation and is also insulin resistant, although not as severely insulin resistant as his daughter (6,74). If the father's second allele of the insulin-receptor gene is normal, then it is possible to conclude that the phenotype of insulin resistance caused by this non-

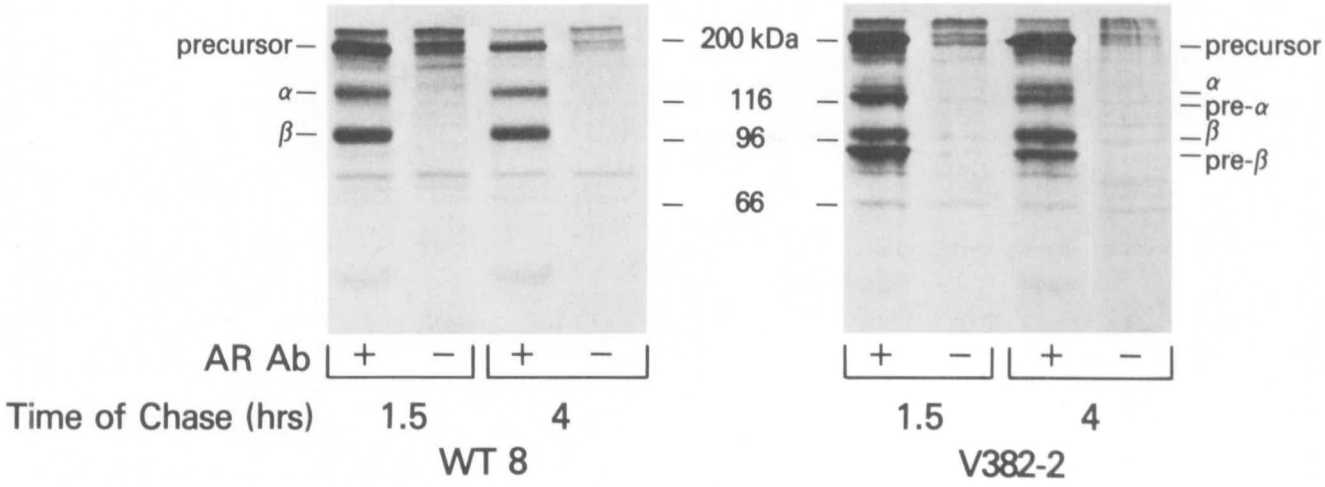

FIG. 8. Val[382] mutation impairs posttranslational processing of insulin receptor. Transfected cells expressing either wild-type insulin-receptor cDNA (WT8) or mutant receptor cDNA (V382-2) were pulse labeled with [^{35}S]methionine for 3 h followed by a chase with unlabeled methionine for either 1.5 or 4 h (12). Cells were solublized and receptors immunoprecipitated. Fluorographs of the sodium dodecyl sulfate–polyacrylamide gels are shown. Molecular weight of receptor precursor (190,000 M_r) and mature receptor α- and β-subunits (130,000 and 100,000 M_r, respectively) together with molecular-weight size markers are indicated on *left* of figure. Molecular weights of pre-α- and pre-β-precursor (120,000 and 90,000 M_r, respectively) are indicated on *right*.

sense mutation is inherited in a codominant manner (6). Consistent with this conclusion, there is a 60–70% decrease in ^{125}I-insulin binding to the father's circulating monocytes (Fig. 10). This suggests that the normal allele of the insulin-receptor gene does not increase the level of expression to compensate for the nonsense mutation.

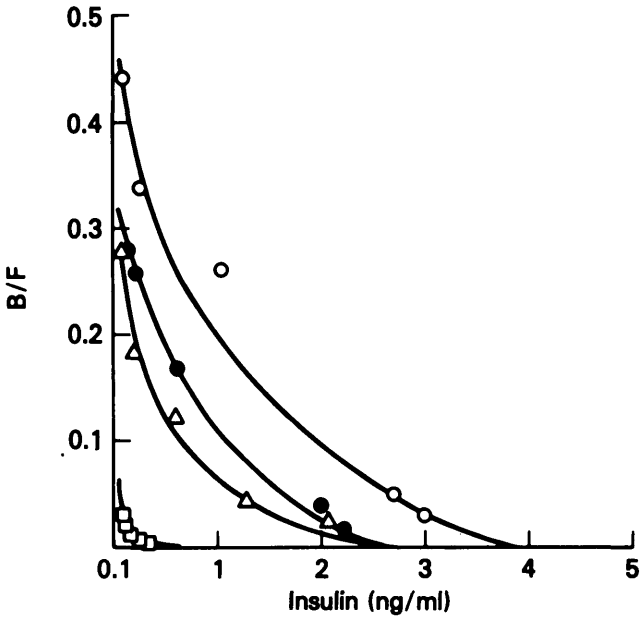

FIG. 9. Val[382] mutation decreases number of insulin receptors on cell surface. Scatchard plot of ^{125}I-insulin binding to transfected cells expressing wild-type (△, WT 2; ○, WT 8) or mutant (□, V382-1; ●, V382-2) insulin-receptor cDNA (12). Three cell lines (△, ○, and □) expressed ~2000 insulin-receptor mRNA molecules/cell; one cell line (●) expressed ~20,000 insulin-receptor mRNA molecules/cell.

In fact, because the father is hyperinsulinemic with plasma insulin levels elevated 5- to 10-fold above the normal range, it is likely that his receptors become downregulated in vivo, thus exacerbating the decrease in the number of insulin receptors on the surface of his cells (74). The latter conclusion is supported by the observation that the number of insulin receptors on the surface of his EBV-transformed lymphoblasts is in the lower half of the normal range when the cells are cultivated in the absence of insulin, thereby eliminating the contribution due to downregulation (Fig. 10).

Accelerated receptor degradation. In the same patient with leprechaunism (leprechaun/Ark-1) with the nonsense mutation described above, there is a second mutant allele containing a missense mutation that encodes the substitution of glutamic acid for lysine at position 460 in the α-subunit of the insulin receptor (6; Fig. 2). Insulin resistance caused by the Glu[460] mutation is inherited in a recessive fashion in that the mother, who is a heterozygous carrier of this mutation, has normal glucose tolerance and does not appear to be insulin resistant. Before cloning, we identified multiple abnormalities in insulin binding to receptors on the surface of the patient's EBV-transformed lymphoblasts, including decreased sensitivity to changes in temperature and pH (72,74,75; Fig. 11). The patient's receptor had a fivefold increase in binding affinity at physiological temperature and pH (37°C, pH 7.4). Furthermore, insulin stimulates the receptor-associated tyrosine kinase normally in receptors from the patient's EBV-transformed lymphoblasts (73).

How does the Glu[460] mutation impair receptor function to cause insulin resistance in vivo? An analogy to a site-directed mutant of the LDL receptor suggests an answer to the question (79). A portion of the extracel-

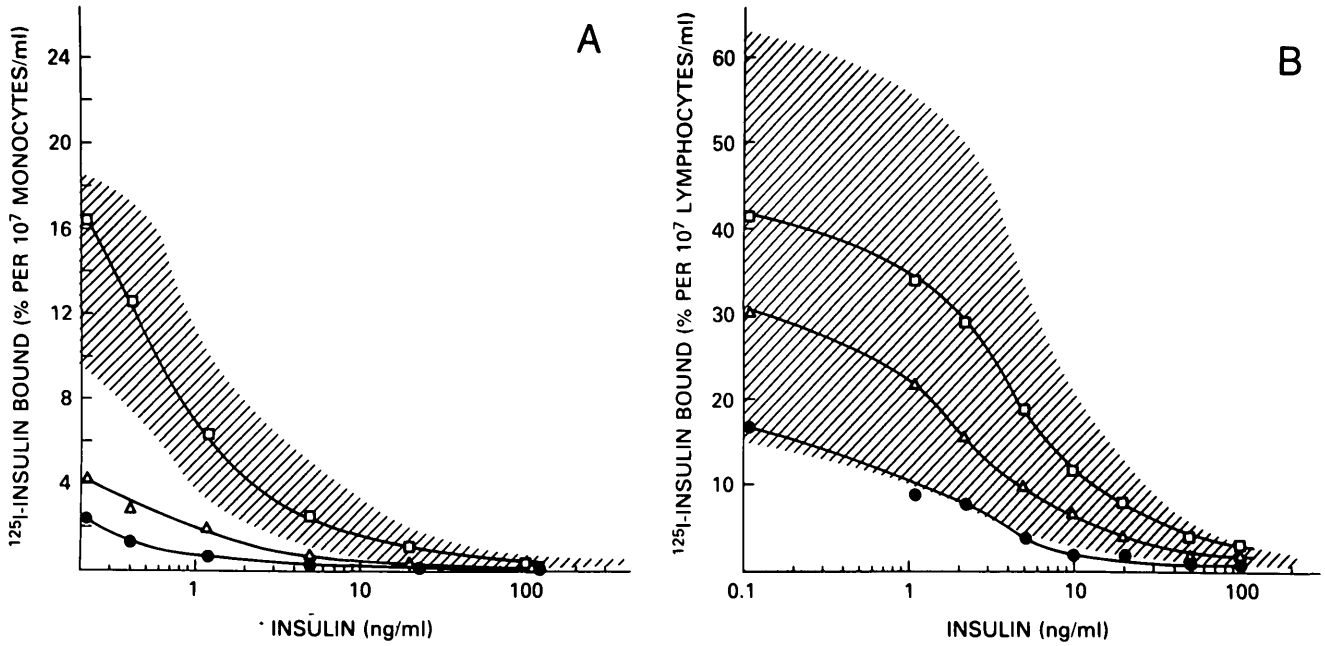

FIG. 10. Decreased ^{125}I-insulin binding to circulating monocytes of patient with leprechaunism (leprechaun/Ark-1; ●) and her father (△). Circulating monocytes (*A*) and Epstein-Barr virus–transformed lymphoblasts (*B*) were incubated with ^{125}I-insulin (0.1 ng/ml) plus varying concentrations of unlabeled insulin. Percentage of ^{125}I-insulin bound specifically is plotted as function of insulin concentration. *Cross hatched area* is range of binding observed in healthy subjects (74).

lular domain of the LDL receptor is homologous to the epidermal growth factor precursor molecule. To investigate the function of this domain of the LDL receptor, a mutant LDL receptor was constructed in which the domain homologous to the epidermal growth factor precursor was deleted. This did not interfere with LDL binding but rendered it insensitive to changes in pH. After the receptors for insulin and LDL (and many other receptors) bind their ligands, the ligand-receptor complex is internalized into endocytic vesicles (Fig. 12). These endocytic vesicles possess proton pumps in their membranes so that they can develop an acidic pH within their lumens. This acidic pH plays a crucial role in dissociating the ligand from its receptor. Subsequent to internalization, at least two distinct pathways are available to the receptor: recycling to the cell surface for reutilization or degradation within the lysosomes. In the case of the deletion mutant of the LDL receptor, desensitization to changes in pH was associated with inhibition of the recycling pathway. This rendered the receptor hypersensitive to ligand-induced downregulation. After binding to the receptor at the cell surface, the ligand was not dissociated from the receptor in the endocytic vesicle, and the receptor was preferentially targeted for degradation within the lysosome (79).

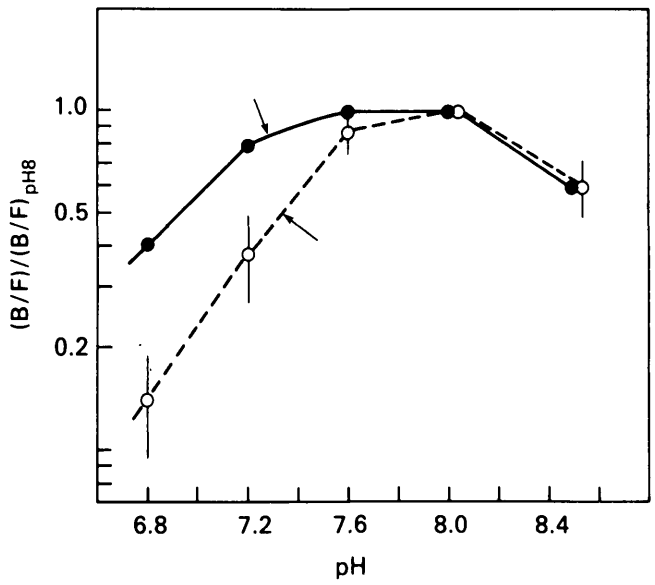

FIG. 11. Abnormal pH sensitivity of ^{125}I-insulin binding to insulin receptors from patient with leprechaunism (leprechaun/Ark-1). ●, pH profile of ^{125}I-insulin binding to Epstein-Barr virus–transformed lymphoblasts of leprechaun/Ark-1; ○, control subjects. Data with control subjects are means ± SE (error *bars*) (72).

The Glu^{460} mutation renders the insulin receptor less sensitive to changes in pH (6,72,74; Fig. 13). With normal insulin receptors (Lys^{460}), decreasing the pH from 7.8 to 6.0 causes a 10-fold acceleration in the rate at which ^{125}I-insulin dissociates from its receptor. The effect of acid pH to accelerate ^{125}I-insulin dissociation is markedly blunted with the Glu^{460} mutant receptor (6; Fig. 13). Analogous to the mutant form of the LDL receptor, the Glu^{460} mutation in the insulin receptor appears to inhibit receptor recycling and accelerate the rate of receptor degra-

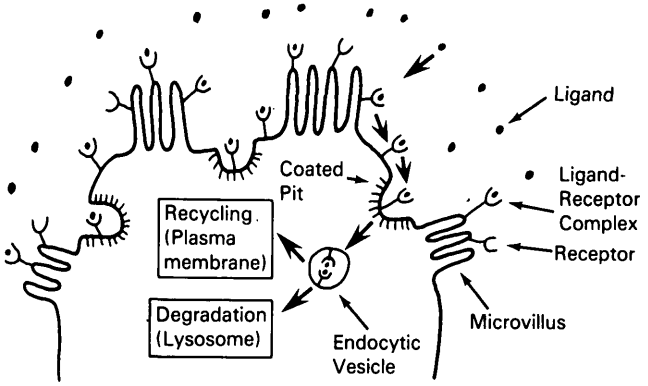

FIG. 12. Pathway of receptor-mediated endocytosis and receptor recycling. After insulin binds to its receptor, hormone-receptor complex undergoes endocytosis. Because of presence of proton pumps in their membranes, endosomes acquire acidic pH in their lumens. Once within cell, 2 alternative fates are available to ligand and its receptor. They can be targeted to lysosome where they will be degraded. Alternatively, the receptor can be recycled back to plasma membrane for reutilization. If ligand escapes degradation, it can be retroendocytosed intact into extracellular fluid.

dation. Several observations support this hypothesis: *1*) insulin receptors on the surface of EBV-transformed lymphoblasts of leprechaun/Ark-1 are degraded almost twice as fast as receptors on the surface of cells from healthy individuals ($t_{1/2}$ = 3.5 h, healthy individuals 6.5 ± 2.4 h, mean ± 2SD) (66); and *2*) the number of insulin receptors on the surface of the patient's circulating monocytes is decreased by 80–85% below the normal range (74).

This hypothesis also suggests resolution of a discrepancy in observations with the patient's EBV-transformed lymphocytes and cultured skin fibroblasts. The number of insulin receptors on the surface of the patient's EBV-transformed lymphocytes was in the low normal range (6,74). In contrast, there was a marked decrease in the number of high-affinity insulin receptors on the surface of the patient's skin fibroblasts (80). Previous studies had shown that the internalization-recycling pathway is inactive in EBV-transformed lymphocytes (81). Therefore, inhibition of the recycling pathway would be predicted to have little impact on the rate of receptor degradation. If receptor recycling were more active in cultured skin fibroblasts, then inhibition of receptor recycling would be predicted to cause a major decrease in the number of insulin receptors on the cell surface.

The number of insulin receptors on the surface of the patient's circulating monocytes was also decreased (74). At least three factors combined to cause this decreased number of receptors on the monocytes. First, the paternal allele of the insulin-receptor gene had a nonsense mutation whereby it encoded a truncated receptor that was not expressed on the cell surface. Second, the receptor encoded by the maternal allele had Glu^{460} substitution which accelerated receptor degradation. Third, the patient had markedly elevated levels of insulin in her plasma; this hyperinsulinemia would be expected to decrease the number of insulin receptors on the cell surface by downregulation.

DEFECTS IN INSULIN-RECEPTOR FUNCTION

In some insulin-resistant patients, there is a normal number of insulin receptors on the cell surface. However, the receptors are defective in their function. At least two activities are necessary for the insulin receptor to perform its essential role in mediating insulin action: insulin binding and tyrosine-specific protein kinase. Mutations that impair both of these functions have been identified.

Mutations decreasing insulin-binding activity. As stated previously, the insulin receptor is synthesized as a single polypeptide precursor that undergoes proteolytic cleavage into two separate subunits. Two sisters with type A extreme insulin resistance have been described who are homozygous for a mutation substituting serine for arginine at position 735, the last amino acid in the tetrabasic amino acid sequence (Arg-Lys-Arg-Arg) in the proteolytic processing site (Fig. 2). This mutation, initially reported by Yoshimasa et al. (7) and subsequently confirmed by Kobayashi et al. (8), prevents proteolytic processing of the receptor precursor into two subunits. As shown in studies of patients' cultured cells, the uncleaved receptor has a decreased affinity to bind insulin (82,83). Presumably, it is the failure of cleavage

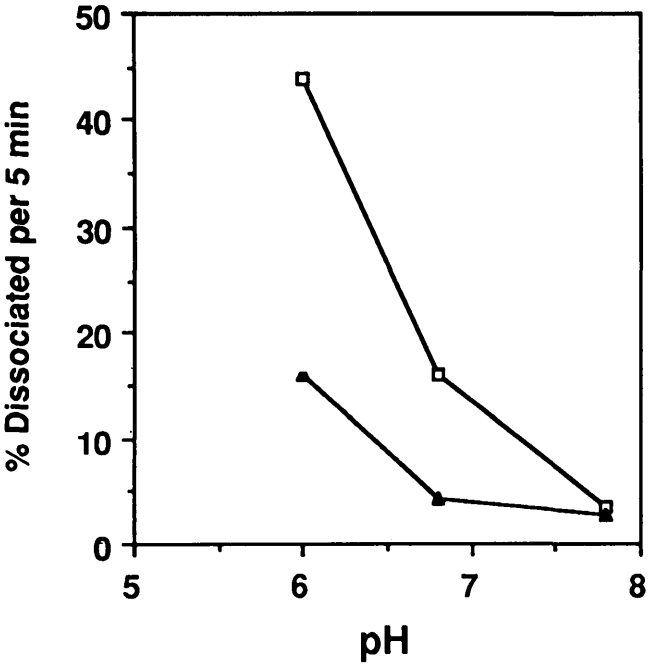

FIG. 13. Glu^{460} mutation decreases sensitivity of insulin receptors to changes in pH. NIH-3T3 cells transfected with either wild-type insulin-receptor cDNA (□; Lys^{460}) or mutant insulin receptor (▲; Glu^{460}) cDNA were incubated with ^{125}I-insulin. Thereafter, unbound ^{125}I-insulin was removed by washing cells at 4°C. Fresh mediums with varying pH (6.0, 6.8, or 7.8) were added, and rate at which ^{125}I-insulin dissociated was measured (6).

into subunits that causes the reduction in binding affinity. However, the possibility has not been ruled out that point mutation causes a decrease in receptor affinity below that which would be observed with the uncleaved precursor having the wild-type sequence.

Recently, another mutation has been identified in the insulin receptor of leprechaun/Geldermalsen: substitution of proline for leucine at position 233 in the α-subunit (13). The patient, who is part of a consanguineous pedigree, is homozygous for the Pro^{233} mutation. The mutation is associated with a marked decrease in insulin binding to the surface of the patient's cultured skin fibroblasts. However, when receptors were solubilized in detergent, the solubilized receptor was stated to bind insulin normally. It is possible that the Pro^{233} mutation decreases insulin binding by preventing transport of the receptors to the cell surface (cf, patients A-5 and A-8) and that detergent increases insulin binding by solubilizing intracellular insulin receptors. Alternatively, it is possible that the mutation causes a decrease in binding affinity when the receptor is on the cell surface, but solubilization with detergent somehow restores the binding affinity to normal. Expression of the mutant receptor cDNA should eventually answer these questions.

Mutations decreasing tyrosine kinase activity. There is evidence that supports the hypothesis that tyrosine-specific protein kinase is necessary for the insulin receptor to mediate insulin action (44–46,48,49,56). Consistent with this hypothesis, several patients have been described in whom insulin resistance is caused by defects in the insulin-receptor tyrosine kinase activity (9,10,84). We cloned insulin-receptor cDNA from one such patient (10). In that patient, we identified a missense mutation that encodes substitution of valine for Gly^{1008}, the third glycine in the Gly-X-Gly-X-X-Gly motif, which is part of the putative ATP binding site (Fig. 2). By transfecting mutant insulin-receptor cDNA into Chinese hamster ovary cells, we have shown that the Val^{1008} mutation abolishes tyrosine kinase activity (10; Fig. 14). The patient's other allele of the insulin-receptor gene had the normal sequence in this region so that the patient was heterozygous for the Val^{1008} mutation. Because the patient's other allele has not been fully sequenced, we do not know whether it is normal or has a second mutation. Thus, we do not know whether the Val^{1008} mutation is dominant or recessive.

Moller and Flier (9) described a variant sequence in which serine is substituted for Trp^{1200} in the β-subunit. This mutation is found in only one allele of the patient's insulin-receptor gene; the other allele is normal in this region. Transfection studies have not yet been reported with the variant form of the insulin receptor containing the Trp^{1200} substitution.

Finally, two patients from Japan have been described who have chromosomal deletions that disrupt the portion of the insulin-receptor gene encoding the β-subunit (11). Because the deletions have been found in only one allele, the mutations appear to be dominant. However, the nucleotide sequence of the second allele has not been determined in these patients. Thus, in addition to point mutations, deletions of portions of the insulin-receptor gene can lead to the synthesis of defective insulin receptors, thereby causing insulin resistance. Of course, in evaluating the significance of deletions of large portions of the receptor, it must be emphasized that multiple receptor functions may be compromised in addition to the defect in tyrosine kinase activity.

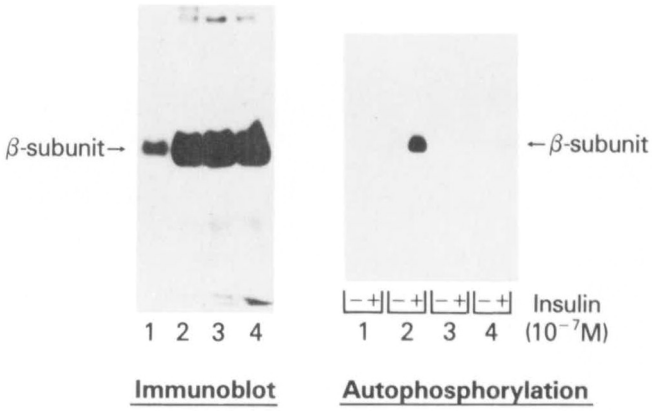

FIG. 14. Expression of normal and mutant human insulin-receptor cDNA in Chinese hamster ovary (CHO) cells. CHO cells were stably transfected with either normal insulin-receptor cDNA or cDNA encoding Gly^{1008}-mutant form of receptor(10). Protein (immunoblot) blotting of partially purified insulin receptors (*left panel*). Site-specific antibody (Ab-IRC) directed against COOH-terminal of human insulin-receptor β-subunit was used to quantitate number of insulin receptors in cells: nontransfected CHO cells (*lane 1*); cells transfected with normal human insulin-receptor cDNA (Gly^{1008}; *lane 2*); two cell lines transfected with mutant insulin-receptor cDNA (Val^{1008}; *lanes 3* and *4*). Based on insulin-binding studies, we estimate that transfected cell lines have 30,000–50,000 receptors/cell. In contrast, nontransfected cells have ~1500 receptors cell. Autophosphorylation (*right panel*) of partially purified insulin receptors from nontransfected cells, cells transfected with normal human insulin-receptor cDNA (Gly^{1008}), or cells transfected with Val^{1008} mutant insulin-receptor cDNA. CHO cells were solubilized in presence of Triton X-100, and receptors were partially purified over wheat germ agglutinin-agarose. After incubation in absence (−) or presence (+) of 10^{-7} M insulin, partially purified receptors (10 μg/lane) were phosphorylated in presence of 50 μM [γ-^{32}P]ATP and 4 mM $MnCl_2$. After immunoprecipitation with Ab-IRC, receptors were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography (10).

NIDDM

Insulin resistance contributes greatly to the pathogenesis of NIDDM (85,86). Furthermore, there is evidence that genetic factors contribute to the predisposition to develop NIDDM. Because the presence of insulin resistance is among the best predictors of future development of NIDDM, it is reasonable to ask whether there is a genetic etiology for insulin resistance in patients with

NIDDM. For target cells to respond to insulin requires the function of many proteins encoded by many genes. Each of these genes is a candidate to be the locus of a mutation causing insulin resistance in patients with NIDDM. In light of the central role of the insulin receptor in mediating the first step in insulin action, it is reasonable to ask whether patients with NIDDM have mutations in the insulin-receptor gene. Supporting this hypothesis, some patients with extreme insulin resistance have had relatives in whom heterozygosity for mutations in the insulin-receptor gene is associated with a moderate degree of insulin resistance. For example, the father of leprechaun/Ark-1, who was heterozygous for a nonsense mutation in the insulin-receptor gene, had a moderate degree of insulin resistance comparable to what is observed in patients with NIDDM (6,74). Furthermore, the mutations detected thus far in the insulin-receptor gene have all caused major defects in the function of the insulin receptor. It is possible that patients with a milder degree of insulin resistance might have mutations that cause less severe disruption of the function of the insulin receptor.

STRATEGY TO IDENTIFY PATIENTS WITH MUTATIONS IN INSULIN-RECEPTOR GENE

Determination of the nucleotide sequence of the insulin-receptor gene requires an expenditure of manpower as well as other resources. Accordingly, before embarking on such a project, there is a strong desire to design a screening test to identify patients who are likely to have mutations in their insulin-receptor genes. Several strategies have been used.

Assays of insulin-receptor function. Thus far, all reported mutations in the insulin-receptor gene have been identified in patients in whom there were identifiable defects in insulin-receptor function, either abnormal insulin binding or impaired tyrosine kinase activity. However, even in patients with definite mutations, some of the functional defects have been subtle. Different laboratories studying the same patients have reached different conclusions. Occasionally, it has been difficult to reconcile seemingly disparate observations obtained with different cell types from the same patient (2,58,60, 72,80,87,88). Although decreased insulin binding (89) and impaired tyrosine kinase activity (90–92) have been described in patients with NIDDM, most evidence suggests that these defects can be reversed with therapeutic intervention such as weight reduction (93). It has been assumed that mutations in the insulin-receptor gene would cause defects in insulin-receptor function that could not be reversed by therapeutic intervention. This assumption may not be correct.

Analysis of RFLPs: population studies. The insulin-receptor gene is a large gene with >100 kb of intervening sequence (22). Accordingly, it is not surprising that many RFLPs have been identified in the insulin-receptor gene (94–105; Table 3). Because the probability of intragenic crossovers occurring within the insulin-receptor gene is estimated as <1%, it is likely that a mutation in the insulin-receptor gene might be in linkage disequilibrium with an RFLP of the gene. If such a mutation played a role in causing NIDDM, an increased frequency of RFLP in the population with NIDDM can be expected. However, several factors might make it difficult for this strategy to succeed in identifying mutant alleles of the insulin-receptor gene:

1. If the mutation arose a long time ago, then a sufficient number of crossovers might have occurred to abolish the linkage disequilibrium.
2. It is possible that there are different mutations in the insulin-receptor gene in different patients. If each mutation were in linkage disequilibrium with a different RFLP, then no single RFLP would become common among patients with NIDDM.
3. If it were necessary to have mutations in a second gene in addition to the insulin-receptor gene to develop NIDDM, then it is possible that many people with mutations in the insulin-receptor gene would not develop diabetes. Consequently, the mutation in the insulin-receptor gene might be common in nondiabetic individuals.
4. Because NIDDM has onset late in life, many individuals who are destined eventually to develop NIDDM may be misclassified as nondiabetic if studied before the development of diabetes.

Furthermore, there are several factors that may cause spurious associations of RFLPs with NIDDM. First and foremost, if many RFLPs are analyzed, spurious associations may arise by random chance. In addition, NIDDM is increased in prevalence in certain racial and ethnic groups. Thus, it is difficult to obtain a nondiabetic control group that is perfectly matched with respect to racial and ethnic origin. Because RFLPs have different prevalences in different racial groups (e.g., Pima Indians vs. Whites), the prevalence of RFLPs may be an indirect reflection of the racial and ethnic origins of patients with NIDDM. For example, among Mexican Americans it has been suggested that the prevalence of NIDDM is highest among individuals with the greatest contribution of genes derived from the Indian gene pool (106). Thus, it would be predicted that patients with NIDDM should have a higher prevalence of RFLPs that are more common in the Indian gene pool. Such an observation would not necessarily implicate a mutation in the insulin-receptor gene as a causal factor in the development of NIDDM.

In light of the complexity of interpreting these association studies, it is not surprising that this approach has not yielded unequivocal conclusions. Some studies have detected weak associations with RFLPs of the insulin-receptor gene with NIDDM in specific racial groups (107,108). Other studies have failed to detect associations (94,101,104).

Analysis of RFLPs: family studies. With RFLPs as markers to identify alleles of the insulin-receptor gene, it is possible to determine in an individual family whether diabetes segregates together with a particular allele of the insulin-receptor gene. Because this is essentially a

TABLE 3
Restriction-fragment–length polymorphisms (RFLPs) of insulin-receptor gene

Enzyme	Probe	Bands (kb)	Prevalence of polymorphic band in population (%)				Refs.
			Whites	Blacks	Pima Indians	Japanese	
*Bam*HI*	2742–4341	7.4	88				94,95
		6.9	12				
Bgl II	2742–4341	23	83				94
		20.0 + 3.4	17				
Dra I (D1)	2742–4341	2.9	38				96
		1.4	62				
Dra I (D2)*	2742–4341	4.1	20				96
		3.6	80				
EcoRI (E1)	0–1011	22	88				97
		20	12				
*Eco*RI (E2)	1926–2476	5.8	56				98
		5.5	44				
*Hin*dIII (H1)	0–1011	6.5	9				97
		1.4	91				
*Hin*dIII (H2)	1599–2961	11.2	61				96
		10.4	39				
*Hin*dIII (H3)*	1599–2961 or 2742–4341	14.0	18				96
		13.5	82				
*Hin*dIII (H4)	2742–4341	23	15				96
		20	85				
Kpn I	2742–4341	15.9	32				99,100
		11.2 + 4.7	68				
Pst I (P1)	0–1011	2.3	17				97
		1.5	83				
Pst I (P2)	334–1011	15.2	88	87			99,100
		13.7	12	13			
Pvu II	1011–5200	4.4				8	101,102
		3.8				92	
Rsa I (R1)	1599–2961	6.8	52	28	48	21	94,101,103
		6.2	48	67	17	74	
		6.4		6			
		3.4			34		
Rsa I (R2)	1599–2961	2.4	23	17	17		94
		2.2	77	63	63		
Rsa I (R3)	1011–5200	3.6				84	101
		Not detected†				16	
Rsa I (R4)	2746–4345	1.9	55	56			99,100
		0.4	45	44			
Rsa I (R5)	334–1011	0.9	87				99,100
		0.3	13				
Sac I/*Sst* I (S1)*	2742–4341	5.8	12	35	100	11	94,95,104
		5.3	88	65	0	89	
Sac I/*Sst* I (S2)	2742–4341	9.4	88	98	98	98	94,104
		7.0 + 2.4	12	2	2	2	
Sac I/*Sst* I (S3)	1599–2961	4.6	66				105
		3.7	4				
		3.6	30				
Stu I	1011–4200	7.2				11	101
		6.6				89	

*Four RFLPs (*Bam*H1, D2, H3, and S1) are in total linkage disequilibrium with one another.
†It seems likely that a band was present, although it was not identified because it probably comigrated with one of the other bands that was visible in blot. If a shorter cDNA had been used as probe, it is likely that the band could have been detected.

statistical approach, large pedigrees with multiple affected individuals are ideally suited for analysis with this approach. The larger the pedigree, the greater the statistical significance of observing data consistent with linkage of diabetes to the insulin-receptor locus. It is possible to pool data from multiple kindreds. However, below are several of the many factors that may make it difficult to demonstrate linkage with this approach:

1. If the mutations causing diabetes are common, this increases the likelihood that both parents may carry different mutant alleles at the same locus. Thus, two diabetic offspring may both inherit mutant alleles of the same gene, although they do not share any alleles in common. According to the usual method of analysis of these data, observation of two diabetic siblings with no alleles in common would be used as evidence against linkage to the insulin-receptor gene. However, in the case where the two siblings have inherited different mutant alleles (one from the father and the other from the mother), this would be the incorrect conclusion.
2. If the mutations causing NIDDM map to different loci in different families, this makes it more difficult to analyze data pooled from multiple kindreds.
3. If it were necessary to have mutations in a second gene in addition to the insulin-receptor gene to develop NIDDM, then some family members may inherit mutations in the insulin-receptor gene but not develop diabetes because they had failed to inherit a mutant allele at another genetic locus.
4. Because NIDDM has onset late in life, many individuals who are destined to eventually develop NIDDM may be misclassified as nondiabetic if studied before the development of diabetes. When the age of onset is as old as it is with NIDDM, there are few pedigrees in which multiple generations are available for investigation and are also old enough to have developed NIDDM.

The difficulty in analyzing the inheritance of RFLPs in families is not cited to discourage the application of this powerful technique. In fact, many of the analytic problems can be addressed (109). However, it is important to recognize that most of the successful applications of this approach have been to map disease-causing genes in diseases with simple monogenic inheritance (110). The complicated genetics of NIDDM makes it more difficult to map the genes causing the disease. Fortunately, maturity-onset diabetes of the young (MODY) is a variant of NIDDM that has simple autosomal-dominant inheritance. Nevertheless, available data suggest that neither MODY nor NIDDM is caused by a mutation in the insulin-receptor gene, at least in families that have been analyzed thus far (111–114). To date, the only successful use of RFLPs to map a diabetogenic mutation to the insulin-receptor locus has been in a consanguineous pedigree with two sisters with the syndrome of type A extreme insulin resistance (12; Figs. 5 and 6). A study conducted in a family with three siblings with lipoatrophic diabetes yielded data that did not support linkage of the disease to the insulin-receptor locus (115).

RNA heteroduplex mapping. This is a biochemical technique to screen for variations from a normal sequence of the insulin-receptor gene. In this approach, a radioactively labeled single-stranded RNA probe encoding the insulin receptor is hybridized with either mRNA or genomic DNA from the patient (116–118). If the nucleotide sequence in the patient's gene is identical to that in the cloned DNA from which the RNA probe is derived, then the probe will form a perfectly double-stranded complex with the patient's mRNA or DNA. If the sequence of the patient's gene differs from that of the cloned DNA, there will be a region in which the probe does not undergo base pairing. Because RNase A preferentially cleaves single-stranded RNA, the enzyme has the potential to cleave the probe at the point of the mismatch. There are two significant limitations to this approach:

1. False negatives. Available data suggest that RNase A will recognize only half of all possible single base pair mismatches (116,118). Because RNase A cleaves after pyrimidines, the enzyme is most likely to recognizes mismatches where the nucleotide in the RNA probe is either C or U at the position of the mismatch or immediately adjacent to the mismatch (116). However, this rule of thumb is not a perfect predictor. Replacement of RNase A digestion with chemical cleavage techniques has been reported to eliminate the problem of false negatives (119).
2. False positives. There is not a unique normal nucleotide sequence encoding the insulin receptor but rather a group of polymorphic normal sequences (Table 4). Thus, most alleles of the insulin-receptor gene will have nucleotide sequences that differ from the cloned cDNA. Because most of the deviations from the sequence of the cloned cDNA are normal polymorphisms, most of the single base pair mismatches detected by RNase protection assays are normal polymorphisms rather than mutations.

This approach has been used to screen for mutations in the insulin-receptor gene (118). In that study, a mutation was detected in the insulin-receptor gene of a patient with type A extreme insulin resistance, and a silent polymorphism was detected in a patient with lipoatrophic diabetes. However, in a patient with leprechaunism (leprechaun/Ark-1), the technique failed to detect two mutations and several other deviations from the sequence of the cloned cDNA (118).

DIRECT DEMONSTRATION OF MUTATIONS BY DETERMINATION OF NUCLEOTIDE SEQUENCE

Multiple strategies have been used to obtain the nucleotide sequence of the insulin-receptor genes of insulin-resistant patients. Questions that must be addressed in designing an experimental approach are:

TABLE 4
Sequence polymorphisms in insulin-receptor gene

Amino acid no.	Amino acid	Codon	Refs.
Confirmed polymorphisms that do not alter amino acid sequence			
−20	Gly	GGG	4–6,12,13,125,127,a,b,c
	Gly	GGA	22
234	Asp	GAC	4–6,12,13,22,125,127,a,b,c
	Asp	GAT	b
276	Gln	CAG	4,6,12,13,118,127,a,b,c
	Gln	CAA	5,22,118,125
519	Asp	GAC	4,6,12,13,125,127,a,b,c
	Asp	GAT	5,6,22,a
523	Ala	GCG	5,6,12,22,125,127,a,b,c
	Ala	GCA	4,13,127,b
642	Phe	TTC	4–6,12,13,22,125,127,a,b,c
	Phe	TTT	6
1058	His	CAC	4–6,9,10,12,13,22,125,127,a,b,c
	His	CAT	9,10,127,a,c
1062	Leu	CTC	4–6,9,12,13,22,125,127,a,b,c
	Leu	CTT	127
Unconfirmed polymorphisms that alter predicted amino acid sequence			
144	Tyr	TAC	4,12,13,22,125,127,a,b,c
	His	CAC	5
421	Ile	ATC	4,6,12,13,22,125,*127,a,b,c
	Thr	ACC	5
465	Gln	CAG	4,6,12,13,22,125,*127,a,b,c
	Lys	AAG	5
873	Val	GTC	5,6,12,13,22,125,127,a,b,c
	Asp	GAC	4
874	Ser	TCC	5,6,12,13,22,125,127,a,b,c
	Thr	ACC	4
1251	Lys	AAG	5,6,9,12,13,22,125,127,a,b,c
	Asn	AAC	4
Unconfirmed polymorphisms that do not alter predicted amino acid sequence			
31	Gly	GGA	4,5,12,13,22,127,a,b,c
	Gly	GGC	125

Included are lists of all known variants that have been observed in protein-coding nucleotide sequence of insulin-receptor gene. A nucleotide sequence was considered to be unconfirmed if it was identified in only single clone and if efforts were not described in publication to rule out possibility of cloning artifacts or sequencing errors. Polymorphic sequences were considered to be confirmed if each nucleotide sequence was identified in DNA derived from ≥2 individuals or if data were reported that ruled out cloning artifacts and sequencing errors. References to literature are listed at right. In addition, we have included unpublished data of T. Kadowaki, H. Kadowaki, and S.I.T.: patient A-1 (1,58), leprechaun/Minn-1 (58), and patient RM-1 (60). To refer to these unpublished data, we have used letters a, b, and c to represent patients A-1, leprechaun/Minn-1, and RM-1, respectively. In some cases, data were presented on two alleles from same patient so that same reference reported two polymorphic nucleotide sequences (6,10,58,60,118,127,a,b,c).

*In ref. 22, it is stated that cDNA cloned by Whittaker et al. (125) confirmed amino acid sequence predicted by Ebina et al. (5) (i.e., Thr^{421} and Lys^{465}) rather than sequence of Ullrich et al. (4) (i.e., Ile^{421} and Gln^{465}). However, this statement was apparently erroneous (S. Seino and G.I. Bell, unpublished observations). In fact, correct sequence of cDNA cloned by Whittaker et al. (125) was identical to that of Ullrich et al. (5) at these two positions (i.e., Ile^{421} and Gln^{465}).

Which nucleic acid will serve as template, genomic DNA, or mRNA? How will the template be copied and amplified? Which experimental technique will be applied to determine nucleotide sequence?

Choice of nucleic acid template: genomic DNA versus mRNA. Because genomic DNA has intervening sequences interrupting the coding sequence, the insulin-receptor gene is longer than the mRNA. Despite this disadvantage, genomic DNA provides several important advantages as a template for genetic investigations. First, if the mutation is located in an intron or regulatory domain of the gene, it may not be possible to identify the mutation in the mRNA. Second, mRNA molecules encoded by two alleles of the insulin-receptor gene may not be present in equal abundance. For example, many nonsense mutations are associated with a marked decrease in the level of mRNA (65,76–78). If such a mutation were present in a heterozygous state, most of the

mRNA would be derived from the allele without the nonsense mutation. Third, the use of reverse transcriptase to synthesize cDNA from mRNA may introduce cloning artifacts as a result of in vitro errors where the enzyme introduces the wrong nucleotide into the cDNA (120). Finally, adequate quantities of genomic DNA can be obtained from leukocytes present in small samples of blood. To obtain mRNA, it is necessary to establish cultured cell lines or obtain larger blood samples.

Despite the many advantages of genomic DNA as template, most of the published mutations in the insulin-receptor gene have been identified in cDNA synthesized from mRNA templates (6,9,10,12,13). In fact, this remains one of the best ways to identify mutations that alter the amino acid sequence of the receptor protein.

Amplification of DNA: cloning versus polymerase chain reaction (PCR). Most of the reported mutations in the insulin-receptor gene have been identified by cloning either cDNA or genomic DNA. Recently, the PCR catalyzed by *Taq* DNA polymerase has enormously reduced the labor required to amplify regions of either genomic DNA or cDNA (121). There are two significant disadvantages of the PCR strategy. First, like reverse transcriptase, the enzyme *Taq* DNA polymerase has an in vitro error rate of ~1 incorrect base/kb (121). Second, PCR has been used primarily to amplify short stretches of DNA. This makes it more difficult to identify polymorphic sequences to differentiate the patient's two alleles of the insulin-receptor gene. Because such polymorphic markers are rare, the greatest likelihood of distinguishing the two alleles occurs if clones are significantly in excess of 1 kb in length. Fortunately, both shortcomings of PCR are overcome to a large extent by the strategy of directly sequencing amplified DNA without cloning into a vector.

Sequencing strategy: individual clones versus direct sequencing of amplified DNA. If PCR has been used to amplify either genomic DNA or cDNA, then there are two alternative strategies to obtain the nucleotide sequence. Undoubtedly the best strategy is to directly sequence the amplified DNA rather than to clone the amplified DNA into a plasmid or bacteriophage vector (9,65,122–124). In addition to the fact that direct sequencing requires less work, there are substantive advantages as well. First, the sequence represents the average sequence of many amplified molecules rather than the sequence of a single cloned molecule. If cloning artifacts have been introduced by either reverse transcriptase or *Taq* DNA polymerase, then it is unlikely that the same artifactual sequence exists in all of the molecules. Thus, the average sequence will tend to eliminate cloning artifacts. Second, it is possible to obtain the sequence of both alleles of the gene in a single sequencing reaction. If the two alleles have different sequences, two bands will be seen at the position in the sequencing ladder where the two alleles differ. Furthermore, if only a single sequence is seen, this suggests that both alleles have the same sequence. (This assumes that both alleles have been amplified with equal efficiency. As discussed earlier, this assumption is likely to be correct if genomic DNA has been used as the template for the amplification. However, even when genomic DNA is used as the template, variations of the nucleotide sequence in the region of the oligonucleotide primers may alter the efficiency of amplification.)

EVALUATION OF SIGNIFICANCE OF VARIATIONS IN NUCLEOTIDE SEQUENCE

It is necessary to first determine whether or not the observed variation in the nucleotide sequence results from a cloning artifact, e.g., introduction of an incorrect nucleotide due to the intrinsic error rate of reverse transciptase, or *Taq* DNA polymerase. In general, the best approach is to attempt to confirm the variant sequence in a second independent clone. The presence of the same sequence in two independent clones greatly reduces the likelihood that the sequence is artifactual. Note that two clones derived from the same PCR may not be independent, because one clone may have served as template for the synthesis of the other. Thus, if PCR has been used, it may be necessary to conduct a second independent amplification to confirm the variant sequence. Once a variant sequence has been preliminarily identified, several different approaches have been used to confirm the variant. Of course, it is possible to repeat the sequencing reactions (6,9,12,65). Alternatively, it is possible to synthesize allele-specific oligonucleotide probes (10,12,65). When hybridization is conducted under selective conditions, each probe will only detect the sequence that is perfectly complementary, although even a single base pair mismatch will prevent hybridization. This approach has the advantage that it facilitates studying numerous DNA samples and is especially useful to trace the inheritance of the variant allele in the pedigree. Finally, changes in the nucleotide sequence can either create or abolish sites for digestion by restriction endonucleases, and these RFLPs can be used as markers for specific alleles (6).

Normal polymorphisms. Assuming that the nucleotide sequence has been confirmed to be a bona fide variant rather than a cloning artifact, it remains to be determined whether it is a normal polymorphism or a functionally significant mutation. As the nucleotide sequences of more alleles of the insulin-receptor gene are determined, a better understanding of the variant sequences that are common in the population is obtained. Thus far, all of the confirmed polymorphisms in the nucleotide sequence have been silent in the sense that they do not alter the predicted amino acid sequence of the receptor protein (Table 4). Furthermore, as originally suggested by Seino et al. (22), some of the variant sequences may have resulted from cloning artifacts.

Genetic linkage and/or epidemiological association studies. One approach to evaluating the functional significance of a variant sequence is to determine whether it is associated with an altered risk of disease. For example, is the variant sequence more or less common in patients with NIDDM compared with nondiabetic sub-

jects? Within a family, does the variant sequence segreagate together with disease? As described previously, there are many technical obstacles to interpreting this type of statistical evidence. Nevertheless, this is a potentially useful approach. In fact, if a mutation were to cause a subtle alteration in receptor function, this type of statistical evidence might be more convincing than data obtained by expression of transfected DNA.

Expression of transfected mutant cDNA. It is relatively straightforward to infer the significance of some mutations (e.g., nonsense mutations, deletions, frame shifts). However, when the amino acid sequence of the receptor is altered, it is important to determine whether the change in amino acid sequence impairs the function of the receptor. The most powerful technique for this purpose is to express mutant cDNA by transfection into cultured cells (6,12,48,49,56,125). This technique allows for the expression of many receptors and facilitates application of the techniques of cell biology and receptor biochemistry to evaluate receptor function. With this approach, it has been possible to elucidate the mechanisms whereby several missense mutations impair receptor function: *1*) the Val^{382} mutation impairs transport of the receptor to the plasma membrane (12); *2*) the Val^{1008} mutation inactivates receptor tyrosine kinase activity (10); and *3*) the Glu^{460} mutation retards the dissociation of insulin from the receptor within the acidic environment of the endocytic vesicle and accelerates the rate of receptor degradation (6).

It is easy to apply this approach based on the expression of transfected cDNA to study mutations that cause major defects in receptor function. However, it seems likely that some patients may have mutations that cause milder defects in receptor function. It may be more difficult to use this approach to demonstrate smaller abnormalities in receptor function.

ESTIMATE OF PREVALENCE OF MUTATIONS IN INSULIN-RECEPTOR GENE

Thus far, existing evidence suggests that the syndrome of leprechaunism is inherited in a recessive fashion (6,13,74). However, some of the heterozygous carriers of mutations that can cause leprechaunism can be shown to be insulin resistant (6,74,126). Thus, the same mutations that cause the phenotype of leprechaunism with recessive inheritance can cause the phenotype of insulin resistance with codominant inheritance. These observations raise the possibility that insulin resistance associated with the carrier state for leprechaunism might be a risk factor that predisposes toward the development of NIDDM. This hypothesis is analogous to the situation with familial hypercholesterolemia where homozygosity for mutations in the LDL-receptor gene is associated with a severe form of hypercholesterolemia, but heterozygosity is associated with a milder version of a similar syndrome. Even assuming that it is true that heterozygosity for mutations in the insulin-receptor gene can predispose to NIDDM, in what fraction of the population with NIDDM can this pathogenetic mechanism be operative?

If it is assumed that one child is born with leprechaunism each year in the U.S., then the incidence of leprechaunism is ~1/4,000,000 live births. Furthermore, assume that each child with leprechaunism has two mutant alleles of the insulin-receptor gene and that a Hardy-Weinberg equilibrium exists. Then the prevalence of mutant alleles for leprechaunism can be calculated as 1/2000 genes in the gene pool, and the prevalence of the carrier state is ~1/1000 individuals. In contrast, ~10% of individuals in the U.S. are ultimately destined to develop NIDDM when they reach the age of maximum risk. If the assumptions underlying the simple calculations were correct, then it can be concluded that the carrier state for leprechaunism is uncommon when compared with the prevalence of a genetic predisposition to NIDDM. However, there are many reasons to suspect that 1/2000 genes in the gene pool may be a gross underestimate of the prevalence of mutant alleles of the insulin-receptor gene.

Homozygosity for some mutant alleles may be lethal in utero. Some mutations have been identified that completely inactivate the ability of the insulin receptor to mediate insulin action. For example, Kadowaki et al. (6,65) described two nonsense mutations that delete the transmembrane anchor and the entire tyrosine kinase domain, and Taira et al. (11) described a deletion that encoded a fusion protein lacking most of the intracellular portion of the receptor including the tyrosine kinase domain. If an embryo were homozygous for this type of mutation, it would lack functional insulin receptors altogether. It is possible that insulin receptors are required at some crucial step in early development so that homozygosity for this type of devastating mutation would be lethal in utero. Consistent with this hypothesis, no patient with a total lack of insulin receptors has ever been reported. In contrast, LDL receptors are not absolutely required during intrauterine life inasmuch as some patients with the homozygous form of familial hypercholesterolemia are born with a total lack of functional LDL receptors.

Thus, there may be fetuses that are homozygous for mutations in the insulin-receptor gene but are not recognized because of spontaneous abortions early in development. To the extent that this leads to an underestimate of the number of homozygotes, it will also lead to an underestimate of the frequency of mutant alleles in the gene pool, especially an underestimate of the mutations that most severely compromise insulin-receptor function.

Possible underestimate of patients with leprechaunism and other syndromes of extreme insulin resistance. Because leprechaunism is a rare syndrome that is usually fatal within the 1st yr of life, it is possible that many patients are not properly diagnosed (18). Furthermore, there are other clinical syndromes such as type A extreme insulin resistance and Rabson-Mendenhall syndrome that are also associated with mutations in the insulin-receptor gene. Some of these clinical syndromes (e.g., type A extreme insulin resistance) appear to be more common than leprechaunism. Including these other

syndromes as well as undiagnosed cases of leprechaunism would lead to a higher estimate of the frequency of mutant alleles of the insulin-receptor gene.

Possible selective disadvantage caused by mutant alleles of insulin-receptor gene. One of the assumptions underlying the Hardy-Weinberg law is that the mutant allele does not affect fertility or impair reproduction. However, this may not be true in the case of mutant alleles of the insulin-receptor gene. For example, it is possible that in some patients type A extreme insulin resistance may be caused by heterozygosity for mutations in the insulin-receptor gene (9). Because type A extreme insulin resistance is associated with hyperandrogenism and polycystic ovary disease syndrome, women who are heterozygous for mutations in the insulin-receptor gene are likely to have a decreased number of children on average. This, too, would lead to an underestimate of the frequency of the mutant allele in the gene pool.

Likelihood that mutations exist that cause less severe impairment of receptor function. Thus far, all of the mutations in the insulin-receptor gene that have been recognized have caused severe defects in receptor function (6–13). It appears likely that mutations may exist that lead to less severe impairment in receptor function. Although these milder mutations may be more difficult to detect, they may be more common than severe mutations. Several possibilities can be given for this type of mutation: *1*) missense mutations in which the amino acid causes a slight defect in receptor function (e.g., slightly decreased binding affinity or slightly reduced tyrosine kinase activity); *2*) mutations in regulatory domains that reduce the level of insulin-receptor gene expression; and *3*) mutations in exon 11 that would be expressed in mRNA molecules that contain exon 11 but not in mRNA molecules that lack exon 11. Such mutations might have more severe impact on tissues (e.g., liver) in which the mRNA molecules containing exon 11 predominate (28,29). Exon 11 is the only exon in the insulin-receptor gene that has been demonstrated to undergo variable splicing, but it is possible that such a process may affect other exons as well.

Thus far, there is a paucity of data on the nucleotide sequences of the insulin-receptor gene in patients with the common form of NIDDM. However, at least in one case of an insulin-resistant Pima Indian, the predicted amino acid sequence of the insulin receptor was normal (127). As the technology to determine nucleotide sequences improves, it is likely that additional data will become available which will allow for a more definitive answer to the question "How common are mutations in the insulin-receptor gene?"

ACKNOWLEDGMENTS

H.K. was supported by a Mentor-Based Fellowship from the American Diabetes Association, and D.A. was supported by a postdoctoral fellowship from the Juvenile Diabetes Foundation International.

We are grateful to Axel Ullrich for providing us with the insulin-receptor cDNA that enabled us to initiate the molecular biological aspects of the work. In addition, we are grateful to Susumu Seino and Graeme I. Bell for generously sharing unpublished information with us. We gratefully acknowledge many colleagues at the National Institutes of Health for helpful discussions and assistance in carrying out this work, especially Jesse Roth, Phillip Gorden, Masato Kasuga, Bernice Marcus-Samuels, Victoria Moncada, and Maxine Lesniak.

REFERENCES

1. Kahn CR, Flier JS, Bar RS, Archer JA, Gorden P, Martin MM, Roth J: The syndromes of insulin resistance and acanthosis nigricans: insulin-receptor disorders in man. *N Engl J Med* 294:739–45, 1976
2. Taylor SI: Receptor defects in patients with extreme insulin resistance. *Diabetes Metab Rev* 1:171–202, 1985
3. Taylor SI: Insulin action and inaction. *Clin Res* 35:459–67, 1987
4. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, Ramachandran J: Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* (Lond) 313:756–61, 1985
5. Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, Ou JH, Masiarz F, Kan YW, Goldfine ID, Roth RA, Rutter WJ: The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. *Cell* 40:747–58, 1985
6. Kadowaki T, Bevins CL, Cama A, Ojamaa K, Marcus-Samuels B, Kadowaki H, Beitz L, McKeon C, Taylor SI: Two mutant alleles of the insulin receptor gene in a patient with extreme insulin resistance. *Science* 240:787–90, 1988
7. Yoshimasa Y, Seino S, Whittaker J, Kakehi T, Kosaki A, Kuzuya H, Imura I, Bell GI, Steiner DF: Insulin-resistant diabetes due to a point mutation that prevents insulin proreceptor processing. *Science* 240:784–87, 1988
8. Kobayashi M, Sasaoka T, Takata Y, Ishibashi O, Sugibayashi M, Shigeta Y, Hisatomi A, Nakamura E, Tamaki M, Teraoka H: Insulin resistance by unprocessed insulin proreceptors point mutation at the cleavage site. *Biochem Biophys Res Commun* 153:657–63, 1988
9. Moller DE, Flier JS: Detection of an alteration in the insulin-receptor gene in a patient with insulin resistance, acanthosis nigricans, and the polycystic ovary syndrome (type A insulin resistance). *N Engl J Med* 319:1526–29, 1988
10. Odawara M, Kadowaki T, Yamamoto R, Shibasaki Y, Tobe K, Accili D, Bevins C, Mikami Y, Matsuura N, Akanuma Y, Takaku F, Taylor SI, Kasuga M: Human diabetes associated with a mutation in the tyrosine kinase domain of the insulin receptor. *Science* 245:66–68, 1989
11. Taira M, Taira M, Hashimoto N, Shimada F, Suzuki Y, Kanatsuka A, Nakamura F, Ebina Y, Tatibana M, Makino H, Yoshida S: Human diabetes associated with a dele-

tion of the tyrosine kinase domain of the insulin receptor. *Science* 245:63–66, 1989
12. Accili D, Frapier C, Mosthaf L, McKeon C, Elbein S, Permutt MA, Ramos E, Lander E, Ullrich A, Taylor SI: A mutation in the insulin receptor gene that impairs transport of the receptor to the plasma membrane and causes insulin resistant diabetes. *EMBO J* 8:2509–17, 1989
13. Klinkhamer M, Groen NA, van der Zon GCM, Lindhout D, Sandkuyl LA, Krans HM, Möller W, Maassen JA: A leucine-to-proline mutation in the insulin receptor in a family with insulin resistance. *EMBO J* 8:2503–07, 1989
14. Flier JS, Minaker KL, Landsberg L: Impaired in vivo insulin clearance in patients with severe target-cell resistance to insulin. *Diabetes* 31:132–35, 1982
15. Taylor SI, Dons RF, Hernandez E, Roth J, Gorden P: Insulin resistance associated with androgen excess in women with autoantibodies to the insulin receptor. *Ann Intern Med* 97:851–55, 1982
16. Barbieri RL, Ryan KJ: Hyperandrogenism, insulin resistance, and acanthosis nigricans syndrome: a common endocrinopathy with distinct pathophysiologic features. *Am J Obstet Gynecol* 147:90–101, 1983
17. Rossini AA, Cahill GF Jr: Lipoatrophic diabetes. In *Endocrinology*. Vol. 2. De Groot LJ, Cahill GF Jr, Martini L, Potts JT Jr, Nelson DH, Steinberger E, Winegrad AI, Eds. New York, Grune & Stratton, 1979, p. 1093–97
18. Rosenberg AM, Haworth JC, Degroot GW, Trevanen CL, Rechler MM: A case of leprechaunism with severe hyperinsulinemia. *Am J Dis Child* 134:170–75, 1980
19. Rabson SM, Mendenhall EN: Familial hypertrophy of pineal body, hyperplasia of adrenal cortex and diabetes mellitus. *Am J Clin Pathol* 26:283–90, 1956
20. West RJ, Leonard JV: Familiar insulin resistance with pineal hyperplasia: metabolic studies and effects of hypophysectomy. *Arch Dis Child* 55:619–21, 1980
21. Yang-Feng TL, Francke U, Ullrich A: Gene for human insulin receptor: localization to site on chromosome 19 involved in pre-B-cell leukemia. *Science* 228:728–31, 1985
22. Seino S, Seino M, Nishi S, Bell GI: Structure of the human insulin receptor gene and characterization of its promoter. *Proc Natl Acad Sci USA* 86:114–18, 1989
23. Mamula PW, Wong KY, Maddux BA, McDonald AR, Goldfine ID: Sequence and analysis of promoter region of human insulin-receptor gene. *Diabetes* 37:1241–46, 1988
24. McKeon C, Moncada V, Barr V, Accili D, Frapier C, Taylor SI: Transcriptional regulation of the human insulin receptor gene (Abstract). *Clin Res* 37:572A, 1989
25. Tewari DS, Cook DM, Taub R: Characterization of the promoter region and 3'-end of the human insulin receptor gene. *J Biol Chem* 264:16238–45, 1989
26. Ojamaa K, Hedo JA, Roberts CT Jr, Moncada VY, Gorden P, Ullrich A, Taylor SI: Defects in human insulin receptor gene expression. *Mol Endocrinol* 2:242–47, 1988
27. Goldstein BJ, Kahn CR: Analysis of mRNA heterogeneity by ribonuclease H mapping: application to the insulin receptor. *Biochem Biophys Res Commun* 159:664–69, 1989
28. Seino S, Bell GI: Alternative splicing of human insulin receptor messenger RNA. *Biochem Biophys Res Commun* 159:312–16, 1989
29. Moller DE, Yokota A, Caro JF, Flier JS: Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol Endocrinol* 3:1263–69, 1989
30. McClain D, Mosthaf L, Ullrich A: Properties of the two naturally occurring alternate forms of the insulin receptor (Abstract). *Diabetes* 38 (Suppl. 2):1A, 1989
31. Hedo JA, Kasuga M, Van Obberghen E, Roth J, Kahn CR: Direct demonstration of glycosylation of insulin receptor subunits by biosynthetic and external labeling: evidence for heterogeneity. *Proc Natl Acad Sci USA* 78:4791–95, 1981
32. Hedo JA, Kahn CR, Hayashi M, Yamada KM, Kasuga M: Biosynthesis and glycosylation of the insulin receptor: evidence for a single polypeptide precursor of the two major subunits. *J Biol Chem* 258:10020–26, 1983
33. Deutsch PJ, Wan CF, Rosen OM, Rubin CS: Latent insulin receptors and possible receptor precursors in 3T3-L1 adipocytes. *Proc Natl Acad Sci USA* 80:133–36, 1983
34. Jacobs SJ, Kull FCJ, Cuatrecasas P: Monensin blocks the maturation of receptors for insulin and somatomedin C: identification of receptor precursors. *Proc Natl Acad Sci USA* 80:1228–31, 1983
35. Hedo JA, Collier E, Watkinson A: Myristyl and palmityl acylation of the insulin receptor. *J Biol Chem* 262:954–57, 1987
36. Herzberg VL, Grigorescu F, Edge AS, Spiro RG, Kahn CR: Characterization of insulin receptor carbohydrate by comparison of chemical and enzymatic deglycosylation. *Biochem Biophys Res Commun* 129:789–96, 1985
37. Collier E, Gorden P: The insulin receptor contains O-linked oligosaccharides (Abstract). *Diabetes* 38 (Suppl. 2):178A, 1989
38. Grunfeld C, Shigenaga JK, Ramachandran J: Urea treatment allows dithiothreitol to release the binding subunit of the insulin receptor from the cell membrane: implications for the structural organization of the insulin receptor. *Biochem Biophys Res Commun* 133:389–96, 1985
39. Pilch PF, Czech MP: Interaction of cross-linking agents with the insulin effector system of isolated fat cells: covalent linkage of ^{125}I-insulin to a plasma membrane receptor protein of 140,000 daltons. *J Biol Chem* 254:3375–81, 1979
40. Waugh SM, DiBella EE, Pilch PF: Isolation of a proteolytically derived domain of the insulin receptor containing the major site of cross-linking/binding. *Biochemistry* 28:3448–55, 1989
40a. Yip CC, Hsu H, Patel RG, Hawley DM, Maddux BA, Goldfine ID: Localization of the insulin-binding site to the cysteine-rich region of the insulin receptor α-subunit. *Biochem Biophys Res Commun* 157:321–29, 1988
41. Whittaker J, Thys R, De Meyts P: Mutation of phenylalanine 115 or 116 of the insulin receptor to leucine compromises its binding activity (Abstract). *Diabetologia* 31:559A, 1988
42. Whittaker J, Okamoto A: Secretion of soluble functional insulin receptors by transfected NIH3T3 cells. *J Biol Chem* 263:3063–66, 1988
43. Frias I, Waugh SM: Probing the α–α subunit interface region in the insulin receptor, location of interhalf disulfide(s) (Abstract). *Diabetes* 38 (Suppl. 2):60A, 1989
44. Kasuga M, Karlsson FA, Kahn CR: Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. *Science* 215:185–87, 1982

45. Kasuga M, Zick Y, Blithe DL, Crettaz M, Kahn CR: Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. *Nature* (Lond) 298:667–69, 1982
46. Rosen OM: After insulin binds. *Science* 237:1452–58, 1987
47. Hanks SK, Quinn AM, Hunter T: The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241:42–52, 1988
48. Chou CK, Dull TJ, Russell DS, Gherzi R, Lebwohl D, Ullrich A: Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J Biol Chem* 262:1842–47, 1987
49. Ebina Y, Araki E, Taira M, Shimada F, Mori M, Craik CS, Siddle K, Pierce SB, Roth RA, Rutter WJ: Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proc Natl Acad Sci USA* 84:704–08, 1987
50. Rees-Jones RW, Taylor SI: An endogenous substrate for the insulin receptor-associated tyrosine kinase. *J Biol Chem* 260:4461–67, 1985
51. Perrotti N, Accili D, Marcus-Samuels B, Rees-Jones RW, Taylor SI: Insulin stimulates phosphorylation of a 120-kDa glycoprotein substrate (pp120) for the receptor-associated protein kinase in intact H-35 hepatoma cells. *Proc Natl Acad Sci USA* 84:3137–40, 1987
52. Margolis RN, Taylor SI, Seminara D, Hubbard AL: Identification of pp120, an endogenous substrate for the hepatocyte insulin receptor tyrosine kinase, as an integral membrane glycoprotein of the bile canalicular domain. *Proc Natl Acad Sci USA* 85:7256–59, 1988
53. White MF, Maron R, Kahn CR: Insulin rapidly stimulates tyrosine phosphorylation of a M_r-185,000 protein in intact cells. *Nature* (Lond) 318:183–86, 1985
54. Tornqvist HE, Gunsalus JR, Nemenoff RA, Frackelton HR, Pierce MW, Avruch J: Identification of the insulin receptor tyrosine residues undergoing insulin-stimulated phosphorylation in intact rat hepatoma cells. *J Biol Chem* 263:350–59, 1988
55. White MF, Shoelson SE, Keutmann H, Kahn CR: A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *J Biol Chem* 263:2969–80, 1988.
56. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter WJ: Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* 45:721–32, 1986
57. White MF, Livingston JN, Backer JM, Lauris V, Dull TJ, Ullrich A, Kahn CR: Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. *Cell* 54:641–49, 1988
58. Taylor SI, Samuels B, Roth J, Kasuga M, Hedo JA, Gorden P, Brasel DE, Pokora T, Engel RR: Decreased insulin binding in cultured lymphocytes from two patients with extreme insulin resistance. *J Clin Endocrinol Metab* 54:919–30, 1982
59. Bar RS, Muggeo M, Kahn CR, Gorden P, Roth J: Characterization of the insulin receptors in patients with the syndromes of insulin resistance and acanthosis nigricans. *Diabetologia* 18:209–16, 1980
60. Taylor SI, Underhill LH, Hedo JA, Roth J, Serrano-Rios M, Blizzard RM: Decreased insulin binding to cultured cells from a patient with the Rabson-Mendenhall syndrome: dichotomy between studies with cultured lymphocytes and cultured fibroblasts. *J Clin Endocrinol Metab* 56:856–61, 1983
61. Scarlett JA, Kolterman OG, Moore P: Insulin resistance and diabetes due to a genetic defect in insulin receptors. *J Clin Endocrinol Metab* 55:123–32, 1982
62. Hedo JA, Moncada VY, Taylor SI: Insulin receptor biosynthesis in cultured lymphocytes from insulin-resistant patients. *J Clin Invest* 76:2355–61, 1985
63. Muller-Wieland D, Taub R, Tewari DS, Kriauciunas KM, Sethu S, Reddy K, Kahn CR: Insulin-receptor gene and its expression in patients with insulin resistance. *Diabetes* 38:31–38, 1989
64. Reddy SS-K, Lauris V, Kahn CR: Insulin receptor function in fibroblasts from patients with leprechaunism. *J Clin Invest* 82:1359–65, 1988
65. Kadowaki T, Kadowaki H, Taylor SI: A nonsense mutation causing decreased levels of insulin receptor mRNA: detection by a simplified technique for direct sequencing of genomic DNA amplified by polymerase chain reaction. *Proc Natl Acad Sci USA*. In press
66. McElduff A, Hedo JA, Taylor SI, Roth J, Gorden P: Insulin receptor degradation is accelerated in cultured lymphocytes from patients with genetic syndromes of extreme insulin resistance. *J Clin Invest* 74:1366–74, 1984
67. Moncada VY, Hedo JA, Serrano-Rios M, Taylor SI: Insulin-receptor biosynthesis in cultured lymphocytes from an insulin-resistant patient (Rabson-Mendenhall Syndrome): evidence for defect before insertion of receptor into plasma membrane. *Diabetes* 35:802–807, 1986
68. Yamamoto T, Bishop RW, Brown MS, Goldstein JL, Russell DW: Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbit. *Science* 232:1230–37, 1986
69. Hobbs HH, Brown MS, Goldstein JL, Russell DW: Deletion of the exon encoding cysteine-rich repeat of low-denisty lipoprotein receptor alters its binding specificity in a subject with familial hypercholesterolemia. *J Biol Chem* 261:13114–20, 1986
70. Lander ES, Botstein D: Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* 236:1567–70, 1987
71. Ronnett GV, Tennekoon G, Knutson VP, Lane MD: Kinetics of insulin receptor transit to and removal from the plasma membrane. *J Biol Chem* 258:283–90, 1983
72. Taylor SI, Roth J, Blizzard RM, Elders MJ: Qualitative abnormalities in insulin binding in a patient with extreme insulin resistance: decreased sensitivity to alterations in temperature and pH. *Proc Natl Acad Sci USA* 78:7157–61, 1981
73. Cama A, Taylor SI: Tyrosine kinase activity of insulin receptors from an insulin-resistant patient with leprechaunism. *Diabetologia* 30:631–37, 1987
74. Taylor SI, Marcus-Samuels B, Ryan-Young J, Leventhal S, Elders MJ: Genetics of the insulin receptor defect in a patient with extreme insulin resistance. *J Clin Endocrinol Metab* 62:1130–35, 1986
75. Taylor SI, Leventhal S: Defect in cooperativity in insulin receptors from a patient with a congenital form of extreme insulin resistance. *J Clin Invest* 71:1676–85, 1983
76. Atweh GF, Brickner HE, Zhu X-X, Kazazian HH Jr, Forget BG: New amber mutation in a β-thalassemic gene

with nonmeasurable levels of mutant messenger RNA in vivo. *J Clin Invest* 82:557–61, 1988
77. Fojo SS, Stalenhoef AFH, Marr K, Gregg RE, Ross RS, Brewer HB Jr: A deletion mutation in the ApoC-II gene of a patient with a deficiency of apolipoprotein C-II. *J Biol Chem* 263:17913–16, 1988
78. Urlaub G, Mitchell PJ, Ciudad CJ, Chasin LA: Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. *Mol Cell Biol* 9:2868–80, 1989
79. Davis CG, Goldstein JL, Sudhof TC, Anderson RG, Russell DW, Brown MS: Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* (Lond) 326:760–65, 1987
80. Elsas LJ, Endo F, Strumlauf E, Elders J, Priest JH: Leprechaunism: an inherited defect in a high-affinity insulin receptor. *Am J Hum Genet* 37:73–88, 1985
81. Carpentier JL, Dayer JM, Lang U, Silverman R, Orci L, Gorden P: Down-regulation and recycling of insulin receptors: effect of monensin on IM-9 lymphocytes and U-937 monocyte-like cells. *J Biol Chem* 259:14190–95, 1984
82. Kakehi T, Hisatomi A, Kuzuya H, Yoshimasa Y, Okamoto M, Yamada K, Nishimura H, Kosaki A, Nawata H, Umeda F, Ibayashi H, Imura H: Defective processing of insulin receptor precursor in cultured lymphocytes from a patient with extreme insulin resistance. *J Clin Invest* 81:2020–22, 1988
83. Kobayashi M, Sasaoka T, Takata Y, Hisatomi A, Shigeta Y: Insulin resistance by uncleaved insulin proreceptor: emergence of binding site by trypsin. *Diabetes* 37:653–56, 1988
84. Grunberger G, Zick Y, Gorden P: Defect in phosphorylation of insulin receptors in cells from an insulin-resistant patient with normal insulin binding. *Science* 223:932–34, 1984
85. Reaven GM: Banting Lecture 1988: role of insulin resistance in human disease. *Diabetes* 37:1595–607, 1988
86. DeFronzo RA: Lilly Lecture 1987: the triumvirate: beta-cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes* 37:667–87, 1988
87. Kobayashi M, Olefsky JM, Elders M, Mako ME, Given BD, Schedewie HK, Fiser RH, Hintz RL, Horner JA, Rubenstein AH: Insulin resistance due to a defect distal to the insulin receptor: demonstration in a patient with leprechaunism. *Proc Natl Acad Sci USA* 75:3469–73, 1978
88. Kahn CR, Podskalny JM: Demonstration of a primary (? genetic) defect in insulin receptors in fibroblasts from a patient with the syndrome of insulin resistance and acanthosis nigricans type A. *J Clin Endocrinol Metab* 50:1139–41, 1980
89. Grunberger G, Taylor SI, Donas RF, Gorden P: Insulin receptors in normal and disease states. *Clin Endocrinol Metab* 12:191–219, 1983
90. Comi RJ, Grunberger G, Gorden P: Relationship of insulin binding and insulin-stimulated tyrosine kinase activity is altered in type II diabetes. *J Clin Invest* 79:453–62, 1987
91. Caro JF, Ittoop O, Pories WJ, Meelheim D, Flickinger EG, Thomas F, Jenquin M, Silverman JF, Khazanie PG, Sinka MK: Studies on the mechanism of insulin resistance in the liver from humans with noninsulin-dependent diabetes: insulin action and binding in isolated hepatocytes, insulin receptor structure, and kinase activity. *J Clin Invest* 78:249–58, 1986
92. Freidenberg GR, Henry RR, Klein HH, Reichart DR, Olefsky JM: Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetic subjects. *J Clin Invest* 79:240–50, 1987
93. Freidenberg GR, Reichart D, Olefsky JM, Henry RR: Reversibility of defective adipocyte insulin receptor kinase activity in non-insulin-dependent diabetes mellitus: effect of weight loss. *J Clin Invest* 82:1398–406, 1988
94. Elbein SC, Corsetti L, Ullrich A, Permutt MA: Multiple restriction fragment length polymorphisms at the insulin receptor locus: a highly informative marker for linkage analysis. *Proc Natl Acad Sci USA* 83:5223–27, 1986
95. Elbein SC: Molecular and clinical characterization of an insertional polymorphism of the insulin-receptor gene. *Diabetes* 38:737–43, 1989
96. Sten-Linder M, Stern I, Bell GI, Luthman H: Human insulin receptor RFLPs detected by *Hin*dIII and *Dra* I. *Nucleic Acids Res* 17:1277, 1989
97. Sanna MA, Bell GI, Cao A, Pirastu M: Three RFLPs for the insulin receptor gene INSR: *Eco*RI, *Pst* I, *Hin*dIII. *Nucleic Acids Res* 14:6776, 1986
98. Accili D, Elbein S, McKeon C, Taylor SI: A new *Eco*RI polymorphism for the insulin receptor gene. *Nucleic Acids Res* 17:821, 1989
99. Cox NJ, Spielman RS, Kahn CR, Muller-Wieland D, Kriauciunas KM, Taub R: Four RFLP's of the human insulin receptor gene: *Pst*I, *Kpn*I, *Rsa*I (2 RFLPs). *Nucleic Acids Res* 16:8204, 1988
100. Cox NJ, Spielman RS, Kahn CR, Muller-Wieland D, Kriauciunas KM, Taub R: Four RFLP's of the human insulin receptor gene: *Pst*I, *Kpn*I, *Rsa*I (2 RFLPs). *Nucleic Acids Res* 177:820, 1989
101. Takeda J, Seino Y, Yoshimasa Y, Fukumoto H, Koh G, Kuzuya H, Imura H, Seino S: Restriction fragment length polymorphism (RFLP) of the human insulin receptor gene in Japanese: its possible usefulness as a genetic marker. *Diabetologia* 29:667–69, 1986
102. Takeda J, Seino Y, Fukumoto H, Koh G, Imura H, Bell GI: *Pvu*II polymorphic sites in the human insulin receptor gene INSR. *Nucleic Acids Res* 14:6777, 1986
103. Shaw DJ, Bell GI: *Rsa*I polymorphism at the insulin receptor locus (INSR) on chromosome 19. *Nucleic Acids Res* 13:8661, 1985
104. Li SR, Oelbaum RS, Stocks J, Galton DJ: DNA polymorphisms of the insulin receptor gene in Japanese subjects with non-insulin-dependent diabetes mellitus. *Hum Hered* 38:273–76, 1988
105. Patel P, O'Rahilly S, Ullrich A, Turner RC, Wainscoat JS: A new *Sst*I RFLP associated with human insulin receptor locus. *Nucleic Acids Res* 16:5700, 1988
106. Chakraborty R, Ferrell RE, Stern MP, Haffner SM, Hazuda HP, Rosenthal M: Relationship of prevalence of noninsulin-dependent diabetes mellitus to Amerindian admixture in the Mexican Americans of San Antonio, Texas. *Genet Epidemiol* 3:435–54, 1986
107. McClain DA, Henry RR, Ullrich A, Olefsky JM: Restriction-fragment-length polymorphism in insulin-receptor gene and insulin resistance in NIDDM. *Diabetes* 37:1071–75, 1988
108. Xiang KS, Cox NJ, Sanz N, Huang P, Karam JH, Bell GI: Insulin receptor and apolipoprotein genes contribute to development of NIDDM in Chinese Americans. *Diabetes* 38:17–23, 1989
109. Lander ES, Botstein D: Strategies for studying heterogeneous genetic traits in humans by using a linkage map

of restriction fragment length polymorphisms. *Proc Natl Acad Sci USA* 83:7353–57, 1986
110. Orkin SH: Reverse genetics and human disease. *Cell* 47:845–50, 1986
111. Elbein SC, Borecki I, Corsetti L, Fajans SS, Hansen AT, Nerup J, Province M, Permutt MA: Linkage analysis of the human insulin receptor gene and maturity onset diabetes of the young. *Diabetologia* 30:641–47, 1987
112. O'Rahilly S, Trembath RC, Patel P, Galton DJ, Turner RC, Wainscoat JS: Linkage analysis of the human insulin receptor gene in type 2 (non-insulin-dependent) diabetic families and a family with maturity onset diabetes of the young. *Diabetologia* 31:792–97, 1988
113. Elbein SC, Ward WK, Beard JC, Permutt MA: Familial NIDDM: molecular-genetic analysis and assessment of insulin action and pancreatic β-cell function. *Diabetes* 37:377–82, 1988
114. Cox NJ, Epstein PA, Spielman RS: Linkage studies on NIDDM and the insulin and insulin-receptor genes. *Diabetes* 38:653–58, 1989
115. Kriauciunas KM, Kahn CR, Muller-Wieland D, Reddy SS, Taub R: Altered expression and function of the insulin receptor in a family with lipoatrophic diabetes. *J Clin Endocrinol Metab* 67:1284–93, 1988
116. Myers RM, Larin Z, Maniatis T: Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. *Science* 230:1242–46, 1985
117. Winter E, Yamamoto F, Almoguera C, Perucho M: A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-ras allele in human tumor cells. *Proc Natl Acad Sci USA* 82:7575–79, 1985
118. Goldstein BJ, Kahn CR: Insulin receptor messenger ribonucleic acid sequence alterations detected by ribonuclease cleavage in patients with syndromes of insulin resistance. *J Clin Endocrinol Metab* 69:15–24, 1989
119. Cotton RG, Rodrigues NR, Campbell RD: Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. *Proc Natl Acad Sci USA* 85:4397–401, 1988
120. Sirover MA, Loeb LA: On the fidelity of DNA replication. *J Biol Chem* 252:3605–10, 1977
121. Saiki RK, Gelfand DH, Stoffel S, Sharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–91, 1988
122. Gibbs RA, Nguyen P-N, McBride LJ, Koepf SM, Caskey CT: Identification of mutations leading to the Lesch-Nyhan syndrome by automated direct DNA sequencing of in vitro amplified cDNA. *Proc Natl Acad Sci USA* 86:1919–23, 1989
123. Scharf SJ, Horn GT, Erlich HA: Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* 233:1076–78, 1986
124. Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian HHJ: Characterization of β-thalassemia mutations using direct genomic sequencing of amplified single copy DNA. *Nature* (Lond) 330:384–86, 1987
125. Whittaker J, Okamoto AK, Thys R, Bell GI, Steiner DF, Hofmann CA: High-level expression of human insulin receptor cDNA in mouse NIH3T3 cells. *Proc Natl Acad Sci USA* 84:5237–41, 1987
126. Lekanne Deprez RH, van Loon BJP, van der Zon GCM, Moller W, Lindhout D, Klinkhamer MP, Krans HMJ, Maasen JA: Individuals with only one allele for a functional insulin receptor have a tendency to hyperinsulinemia but not to hyperglycemia. *Diabetologia*. In press
127. Cama A, Patterson AP, Kadowaki T, Kadowaki H, Siegel G, D'Ambrosio D, Lillioja S, Roth J, Taylor SI: The amino acid sequence of the insulin receptor is normal in an insulin resistant Pima Indian. *J Clin Endocrinol Metab*. In press