Human Insulin-Receptor Gene

Partial Sequence and Amplification of Exons by Polymerase Chain Reaction

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The partial sequence of the human insulin-receptor (hINSR) gene is presented. Using the gene sequence as a guide, we selected pairs of oligonucleotide primers from sites in the introns that flank each exon. These primers allowed each of the 22 exons of the hINSR gene to be amplified in vitro by the polymerase chain reaction. The sequences of the gene and oligonucleotide primers will facilitate studies of genetic variation in the hINSR gene and thereby increase our understanding of the role of this gene in the development of insulin-resistant states and glucose intolerance. *Diabetes* 39:123–28, 1990

Recent studies demonstrated that the extreme insulin resistance of the type A syndrome of insulin resistance and acanthosis nigricans, leprechaunism, and the Rabson-Mendenhall syndrome is associated with the biosynthesis of an abnormal insulin receptor (1–8). Eight different mutations in the human insulin-receptor (hINSR) gene have been identified in patients with these syndromes, and it seems likely that they represent the primary genetic lesion responsible for the insulin resistance and other features of these disorders. Patients who have extreme insulin resistance frequently have diabetes mellitus as well, and studies of such patients could provide insight into the role of the hINSR in the development of non-insulin-dependent diabetes mellitus (NIDDM). Moreover, it may also be appropriate to consider the contribution of genetic variation in the hINSR gene to the natural history of the more common forms of NIDDM.

The cloning of hINSR mRNA (9,10) and gene (11) has facilitated the identification and characterization of mutations affecting the function of this receptor. Several approaches are possible for identifying mutations in the hINSR. cDNA and/or genomic libraries could be prepared and used to isolate clones encoding the hINSR. However, the "library approach" is not suitable for screening large numbers of individuals. The polymerase chain reaction (PCR), a technique that allows the specific in vitro amplification of mRNA and DNA segments (12,13), is a relatively easy and powerful alternative that can be adapted to screening large numbers of individuals. Kobayashi et al. (2) and Moller and Flier (4) have used PCR to amplify segments of hINSR mRNA and identify nucleotide substitutions resulting in the synthesis of an abnormal protein in two patients with extreme insulin resistance. Our isolation and characterization of the hINSR gene has allowed us to develop a complementary approach that permits the identification of mutations in the gene that result in the expression of an abnormal mRNA or receptor protein (11). Using the partial sequence of the hINSR gene as a guide, we selected pairs of primers from the flanking introns that allowed us to specifically amplify each of the 22 exons of the hINSR gene. Thus, it is now possible to examine the sequence of the protein-coding regions of the gene and the splice acceptor and donor sites that flank each exon.

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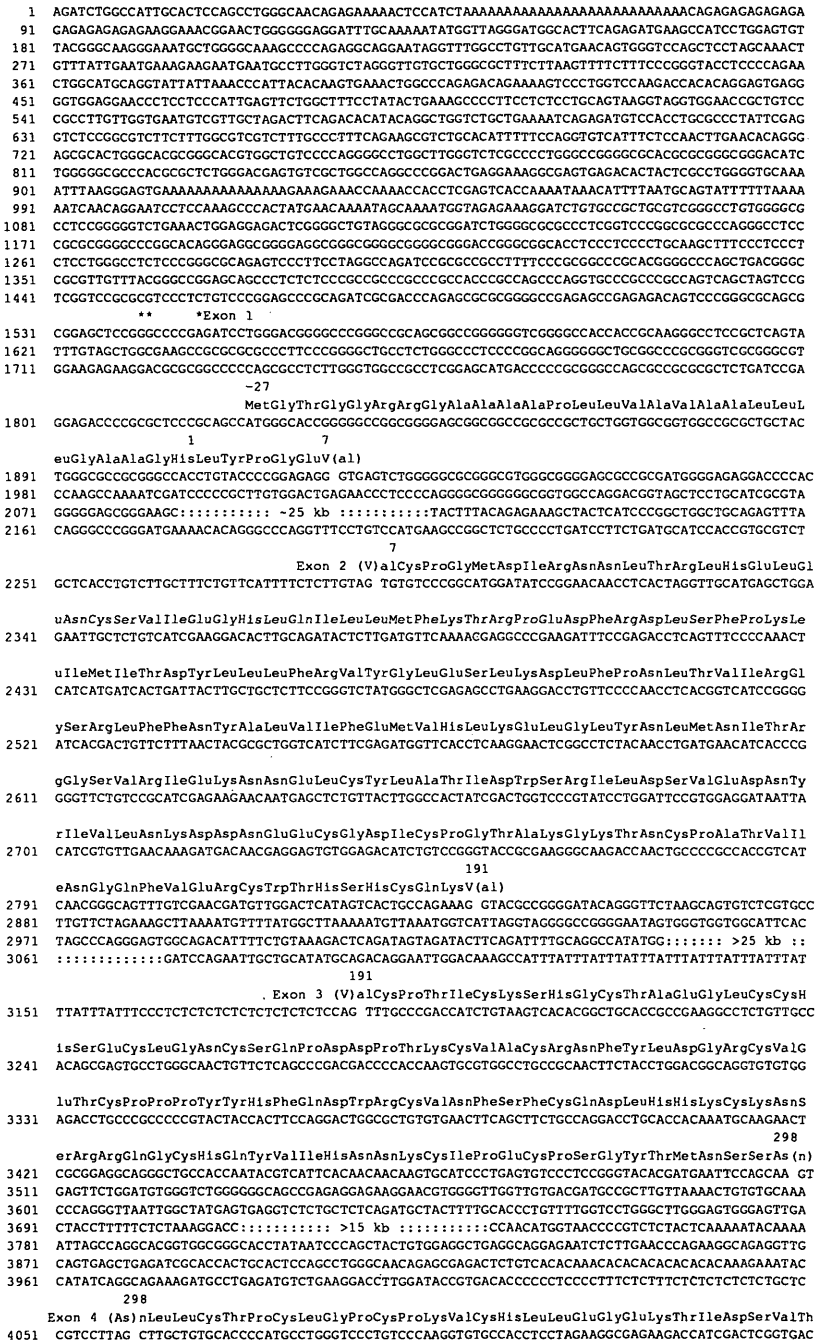
RESEARCH DESIGN AND METHODS

The isolation and characterization of the hINSR gene have been described previously (11). DNA sequencing was done by the dideoxynucleotide chain-termination procedure (14) after subcloning appropriate DNA fragments into M13mp18 or M13mp19. The universal primer and sequence-specific oligonucleotides were used as primers.

In vitro amplification of hINSR exons and adjacent introns. The regions representing exons 2–22 were amplified with standard conditions (12). Each reaction was performed in a volume of 100 μl containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 100 μg/ml gelatin; 200 μM each of dATP, dGTP, dCTP, and dTTP; 1 μM of each oligonucleotide primer (Table 1); 0.5 μg genomic DNA; and 2.5



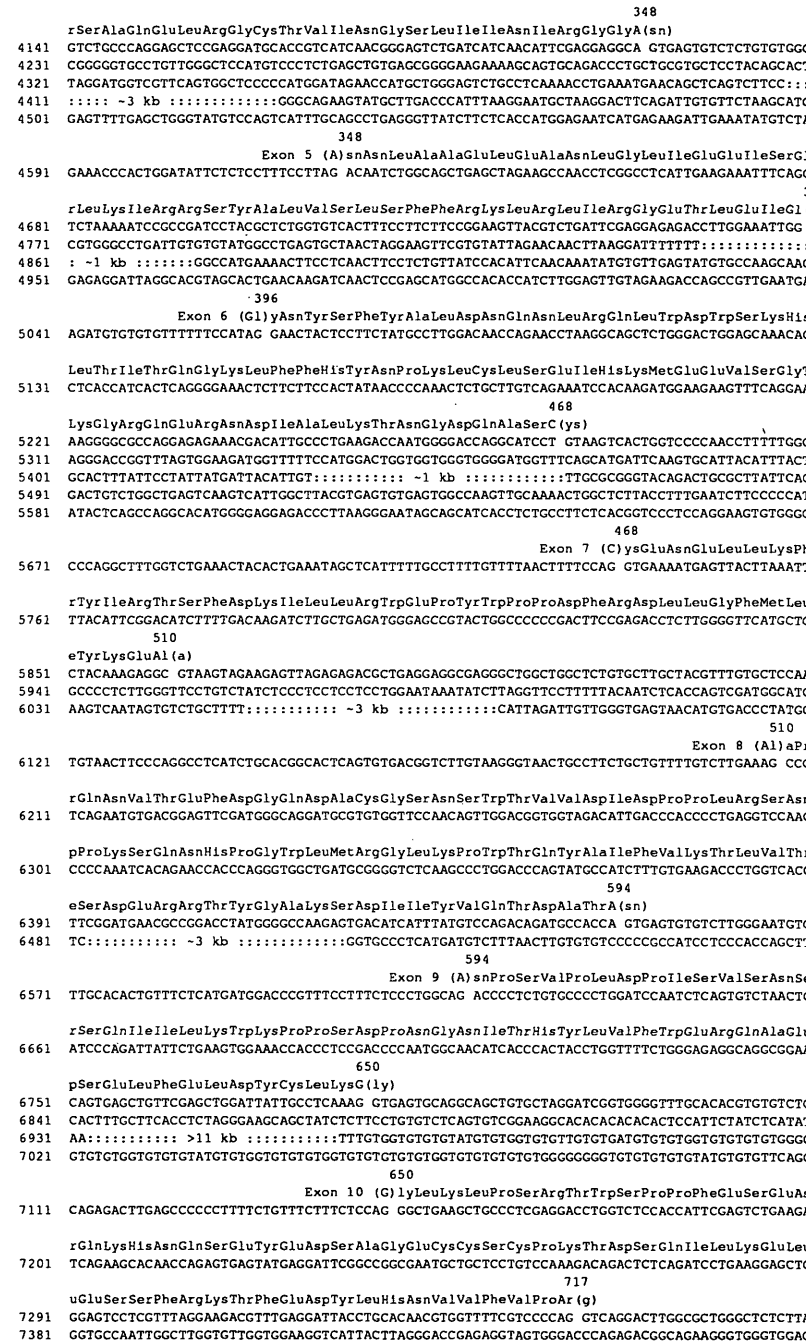


FIG. 1. Partial sequence of human insulin-receptor gene. Nucleotide and predicted amino acid sequence are shown. Each exon is indicated; by convention, introns begin with dinucleotide GT and end with AG. Number of amino acid at beginning and end of exon is noted. Approximate sizes of introns are indicated. Sequence of intron upstream from exon 5 differs by 1 nucleotide from that previously reported (11) because of clerical error in latter. For purposes of discussion, number of nucleotide at beginning of each line is shown; numbering system used includes each character or space, of which there are 90 per line. *Transcriptional initiation sites described in ref. 11 (line beginning with nucleotide 1531).

TABLE 1
Amplification of exon 1 by polymerase chain reaction (PCR)

1. Digest 5 μg of DNA with restriction endonuclease *Sma* I in a volume of 100 μl according to supplier's recommendations.
2. Stop digest by adding 10 μl 3 M sodium acetate and 330 μl ethanol. Collect ethanol precipitate by centrifugation in a microfuge for 10 min. Rinse DNA pellet with 100% ethanol and dry.
3. Resuspend DNA pellet in 90 μl H_2O and then add 10 μl 2 N NaOH and heat at 70°C for 10 min to denature the DNA.
4. Add 50 μl 7.5 M ammonium acetate and 450 μl ethanol. After 10 min in a dry ice–ethanol bath, collect precipitated denatured DNA by centrifugation in a microfuge for 10 min. Rinse DNA pellet with 100% ethanol, dry, and resuspend in 20 μl H_2O.
5. PCR reaction 1: mix 10 μl (2.5 μg) denatured DNA and 100 pmol of each primer in nucleotide-buffer solution described in RESEARCH DESIGN AND METHODS except for substitution of 7-deaza-dGTP for dGTP. After initial denaturation at 94°C for 3 min, sample was subjected to 25 rounds of amplification: denaturation, annealing, and extensions steps were 94°C for 1.5 min, 58°C for 1.5 min, and 72°C for 2.5 min, respectively.
6. Extract PCR product with $CHCl_3$ to remove mineral oil and then add 1.4 ml H_2O. Separate PCR product from unincorporated primers and deoxyribonucleotides with a Centricon-100 microconcentrator (Amicon, Danvers, MA). Volume of retentate is ~45 μl.
7. PCR reaction 2: reamplify DNA in 5 μl of retentate for 15 cycles with the same primer pair, the standard nucleotide-buffer solution, including dGTP, and the parameters described in RESEARCH DESIGN AND METHODS.

U *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA). After an initial denaturation at 94°C for 3 min, the samples were subjected to 25 cycles of amplification with a Perkin-Elmer Cetus DNA ThermalCycler: annealing at 55°C for 1.5 min, extension at 70°C for 2.5 min, and denaturation at 94°C for 1 min.

It was not possible to amplify exon 1 with standard conditions. The procedure used to amplify exon 1 is described in Table 1. The dGTP analogue 7-deaza-dGTP (Boehringer Mannheim, Indianapolis, IN) is used in place of dGTP in the first round of amplification. This analogue is often used in the dideoxynucleotide chain-termination sequencing method in the place of dGTP to overcome compression problems due to secondary structure when sequencing GC-rich stretches of DNA (15). 7-deaza-dGTP has been hypothesized to prevent the formation of secondary structure, because alternative Watson-Crick base pairing is precluded due to the presence of the methene group at the N-7 position.

RESULTS

Partial sequence of hINSR gene. The hINSR gene spans a region of >120,000 base pairs (bp) of the short arm of chromosome 19 (11,16). Most of the gene has been isolated as a series of overlapping DNA fragments in the bacteriophage-λ (11). The gene is composed of 22 exons and 21 introns. Exons 1–11, which span >90,000 bp, encode the α-subunit of the receptor, and exons 12–22, which are located together in a region of ~30,000 bp, code for the β-subunit. The exons range in size from 36 bp (exon 11) to >2500 bp (exon 22). The 21 introns, all of which interrupt protein-coding regions of the gene, vary in size from ~500 to >25,000 bp. The actual size of the hINSR gene is uncertain because parts of introns 2, 3, 9, and 10 have not been isolated. Muller-Wieland et al. (17) isolated a fragment of the gene containing both exons 1 and 2, and their data indicate that intron 1 is ~25,000 bp. We sequenced ~13,000 bp of the hINSR gene, including the 5′-flanking promoter region, each exon, and part of each intron (Fig. 1). This sequence is also available from us on diskette for both IBM and Macintosh computers.

As described previously (11), it was not possible to clone all of exon 10 with standard procedures, except from an individual who was identified by chance and had a deletion of ~1200 bp upstream of exon 10 in one hINSR allele. The sequence of intron 9 upstream of exon 10 includes a region of tandem repeats of the dinucleotide TG that extend for >400 bp (data not shown). We suspect that this feature hampered our cloning of intron 9 from chromosomes not having a partial deletion of intron 9.

Amplification of hINSR exons. Using the sequences of the introns flanking each exon as a guide, we selected pairs of oligonucleotide primers that allowed the protein-coding portion of each exon and flanking splice donor and acceptor sites to be amplified in vitro with PCR (Table 2; Fig. 2). Presumably because of its GC-rich character (Fig. 1), which interferes with the replication of this region by *Taq* DNA polymerase, it has been more difficult to amplify the region around exon 1 with our standard procedure. However, we were able to amplify this exon with a two-step procedure in which initial rounds of DNA synthesis were carried out in the presence of the dGTP analogue 7-deaza-dGTP (Table 1). The sequence of exon 1 amplified by this procedure was identical to that determined from the cloned gene, indicating that amplification in the presence of 7-deaza-dGTP does not appear to increase misincorporation by *Taq* polymerase.

The DNA amplification observed with the primer pairs described in Table 2 is very specific, and analysis of the amplified DNA by gel electrophoresis indicated that little nonspecific priming occurred (Fig. 2). We also cloned and sequenced each amplified exon to confirm the specificity of the amplification reaction, and the nucleotide sequence of each of the amplified exons was identical to that of the cloned gene.

DISCUSSION

We sequenced ~13 kilobases (kb) of the hINSR gene, and Elbein (18) reported the sequence of a 7.2-kb segment that includes exons 14–17 and the site of an insertional restriction-fragment–length polymorphism in intron 14. Thus, the sequence of ~20 kb of the hINSR gene has now been determined; however, this represents only ~10–15% of this gene. Inspection of the sequence indicates regions of repeating oligonucleotides upstream of exons 3 (ATTT), 10 (TG), and 21 (TG) (Fig. 1). Such regions are frequently sites of microheterogeneity in size, and it is often possible to distinguish parental alleles because of differences in the number of repeats (19,20).

Using the sequence of the gene as a guide, we were able to select primer pairs that allowed specific amplification of each exon together with adjacent splice acceptor and donor sites. The amplified DNA can be sequenced, digested with restriction endonucleases, or hybridized to allele-specific oligonucleotides to identify nucleotide substitutions in the protein-coding regions or at the splice acceptor and donor sites.

TABLE 2
Primer pairs for amplifying human insulin-receptor exons

Exon	Upstream primer	Downstream primer	Size of PCR fragment (bp)
1	5′-CGCGCTCTGATCCGAGGAGA (1786–805)	5′-AGGGTTCTCAGTCCACAAGC (2002–21)	236
2	5′-CCCTGATCCTTCTGATGCAT (2218–37)	5′-GCTTTCTAGAACAAGGCACGA (2874–94)	677
3	5′-ACAGGAATTGGACAAAGCCAT (3098–118)	5′-AGCAGAGACCTCACTCATAGCCAA (3612–35)	538
4	5′-GCCTGAGATGTCTGAAGGAC (3979–98)	5′-GCCACTGAACGACCATCCTA (4321–40)	362
5	5′-CTCACCATGGAGAATCATGA (4550–69)	5′-CTAATACACGAACTTCCTAG (4806–25)	276
6	5′-AGGCACGTAGCACTGAACA (4960–78)	5′-TGTAATGCACTTGAATCATGCTG (5370–92)	433
7	5′-CACCTCTGCCTTCTCACGGT (5630–49)	5′-AAACGTAGCAAGCACAGAGC (5909–28)	299
8	5′-CGGTCTTGTAAGGGTAACTG (6161–80)	5′-GAATTCACATTCCCAAGACA (6463–82)	322
9	5′-GCACACTGTTTCTCATGATG (6573–92)	5′-AGAGGTGAAGCAAAGTGCAT (6838–57)	285
10	5′-TGTTCAGCCGCAGAGACTTG (7101–20)	5′-CGGTCCCTAAGTAATGACCT (7408–27)	327
11	5′-GTGGTCTGTCTAATGAAGTT (7575–94)	5′-GAATTGGTGAAGCATCTGCT (7793–812)	238
12	5′-TGATGGTGATGGTGTCATCATA (8000–21)	5′-TGTCCTTGGTCAGCCTTGATGT (8357–78)	379
13	5′-GGATCTCATCCAAGAGTTAC (8735–54)	5′-TACTAATAGCACAGTACCTG (9037–56)	322
14	5′-TGGACACTCCCAGATGTGCA (9186–205)	5′-ACCATGCTCAGTGCTAAGCA (9442–61)	276
15	5′-GTGAACTTTGTTGGAAACACATTG (9612–35)	5′-CCTATACCTATATCAAGGCATG (9827–48)	237
16	5′-TCTGCTGGTAAGGGCTGCCA (10191–210)	5′-CTCACTCAATGGTGAAGGCA (10419–38)	248
17	5′-CCAAGGATGCTGTGTAGATAAG (10650–71)	5′-TCAGGAAAGCCAGCCCATGTC (10946–66)	317
18	5′-CTGGTGAGTCGAATCACGGA (11123–42)	5′-AGCGGGTGCTCCACCGAGTA (11317–36)	214
19	5′-GATCCCAGTGCTGCTGAAACAC (11379–400)	5′-ACGGCTCATTATAGACAACTTC (11642–63)	285
20	5′-AGGTTAAGAGCGTGTGAACCT (11811–31)	5′-GAATTCAAGCCCAGCGTCCAT (11998–2018)	208
21	5′-TGTTACTACTATCAACTGTC (12121–40)	5′-ACCTGTAACATACAGCATGC (12392–411)	291
22	5′-ACTCACCCAGGACGTGTCCTTCT (12537–59)	5′-GAACGATCTCTGAACTCCACT (13026–46)	510

Numbers in parentheses indicate location of the primer in the sequence presented in Fig. 1. PCR, polymerase chain reaction; bp, base pairs.

It can also be labeled by nick translation or oligolabeling and used as a probe to localize restriction-fragment–length polymorphisms.

NIDDM is associated with both impaired β-cell function and insensitivity of the target cell to the action of insulin (21,22). Studies of patients expressing an abnormal insulin (23–25) or INSR (1–8) protein indicated that mutations in these genes increase the risk of developing NIDDM. Insulin gene mutations represent a paradigm for β-cell–specific mutations that can contribute to the development of NIDDM, whereas INSR gene mutations are an example of a defect in the insulin-responsive cell that increases the risk of developing this disorder. Population-association studies suggest that genetic variation in the hINSR gene can modify the phenotypic expression of other diabetogenic genes and thereby increase or decrease susceptibility to this disorder

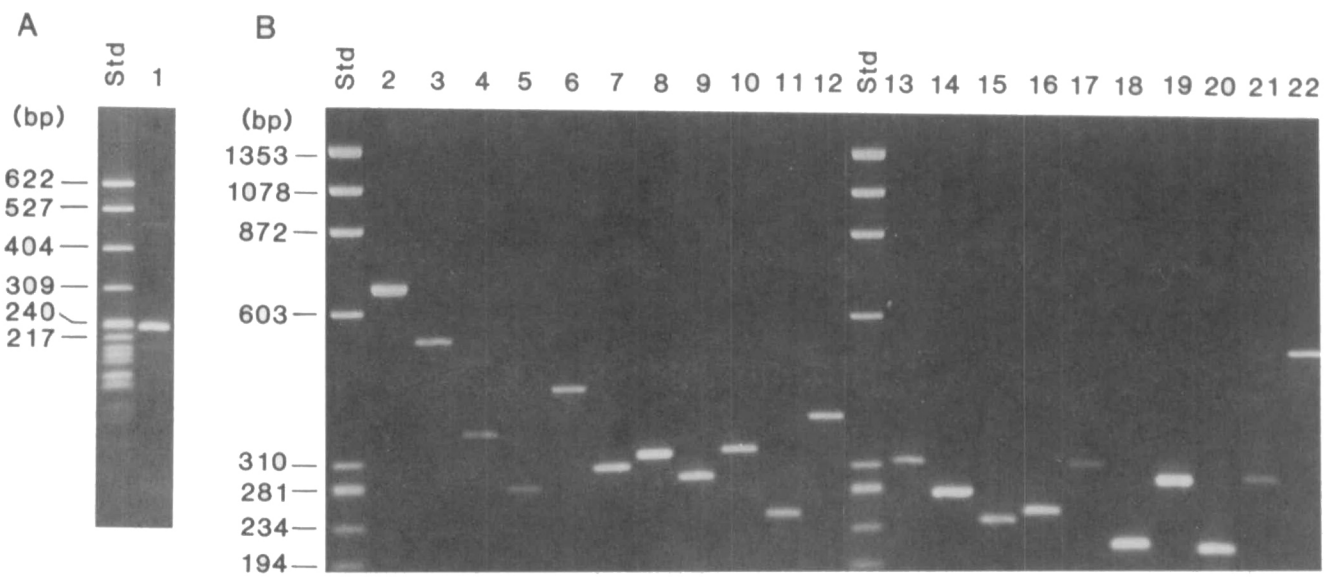


FIG. 2. Amplification of exons 1–22 of human insulin-receptor gene by polymerase chain reaction (PCR). Photographs of ethidium bromide–stained agarose gels of PCR products are shown. Amplified exon (1–22) run in each lane is indicated; *Std*, lanes containing DNA standard. Twenty microliters of each PCR reaction was separated by electrophoresis on 3% NuSieve GTG agarose (FMC, Rockland, ME) gel in Tris-borate buffer. DNA standard used in *A* is *Msp* I digest of pBR322 DNA, and standard used in *B* is *Hae* III digest of ϕX174 DNA. Differences in intensity of ethidium bromide staining are reflection of efficiency of DNA amplification; selection of other primer pairs and/or modification of PCR conditions might increase DNA yield. bp, Base pairs.

(26,27). Thus, the hINSR gene appears to be able to contribute to the development of NIDDM as a primary genetic determinant and a component of the modifying polygenic background. PCR represents a powerful technique that will facilitate studies of genetic variation in the hINSR gene and thereby increase our understanding of the role of this gene in the development of insulin-resistant states and glucose intolerance.

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