Identification of a Point Mutation in the Human Insulin Gene Giving Rise to a Structurally Abnormal Insulin (Insulin Chicago)

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SUMMARY

Both insulin gene alleles of a diabetic patient with a mutant insulin were cloned in a lambda vector and their nucleotide sequences were determined. Nucleotide sequence analysis revealed, in one allele, a C (cytidylate) to G (guanylate) transversion in the codon for phenylalanine at position 25 of the insulin B-chain. This point mutation leads to the substitution of a leucine for phenylalanine accompanied by the loss of a restriction endonuclease MboII recognition site and the creation of a new RsaI cleavage site at this position.

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An abnormal insulin with decreased biologic activity has been identified in a patient (R.C.) with hyperinsulinemic diabetes mellitus.[1,2] Partial characterization of the mutant insulin from this patient indicated that a leucine for phenylalanine substitution had occurred at either position 24 or 25 of the insulin B-chain.[1] Restriction map analysis of leukocyte DNA from this patient with the restriction endonuclease MboII revealed the loss of one MboII recognition site (TCTTC) in the coding sequence of the insulin gene, consistent with the postulated leucine for phenylalanine substitution at either position 24 or 25 of the insulin B-chain.[3] Recently, this mutant insulin (Insulin Chicago) has been identified as Leu^{B25} insulin by high pressure liquid chromatography (HPLC) and radioimmunoassay using semisynthetic human Leu^{B25} insulin as a standard.[4] In addition, two mutant insulins from two unrelated patients also have been characterized.[4] All three mutant insulins have different amino acid substitutions, since they elute at different positions in the HPLC system. Although this method provides a convenient method for detecting and characterizing mutant insulins, the precise identification of the mutation requires that the mutant gene be examined directly. We report here the results of cloning and nucleotide sequence analysis of both the mutant and normal insulin gene alleles from patient R.C., which reveal in the mutant gene a C (cytidylate) to G (guanylate) transversion in the codon for phenylalanine at position 25 of the insulin B-chain, changing this codon to one for leucine. This patient is thus heterozygous for the production of Leu^{B25} insulin.

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MATERIALS AND METHODS

Genomic DNA was prepared from the leukocytes of patient R.C. as described previously.[3] Molecular cloning of the human insulin genes was performed as described in detail elsewhere,[5] except that bacteriophage Charon 4A was used instead of Charon 21A because the former can readily accommodate a 14-kilobase EcoRI-cleaved DNA fragment containing the human insulin gene.

In order to simplify the DNA sequencing work, a 2.6-kilobase BglII/XhoI-digested fragment, which still contained the entire human insulin gene, was obtained from each isolate and subcloned into the BamHI/SalI sites of plasmid pBR322. DNA sequence analysis was performed using the chemical modification method of Maxam and Gilbert.[6]

RESULTS AND DISCUSSION

Preliminary restriction map analysis of the DNA isolated from the leukocytes of patient R.C. showed that restriction endonuclease EcoRI produced a 14-kilobase hybridizing fragment. The 14-kilobase EcoRI-digested DNa fragment was subsequently isolated from an agarose gel, ligated to EcoRI-cleaved Charon 4A phage arms, and packaged in vitro as described elsewhere.[5] The recombinant phages were screened with a human cDNA probe. Of the 610,000 recombinant phages screened, 5 were found to be positive. DNA was isolated from the five positive clones and analyzed by restriction mapping with restriction endonuclease MboII as described previously.[3] Two types of hybridization pattern were observed (Figure 1). Two clones (B and C) produced

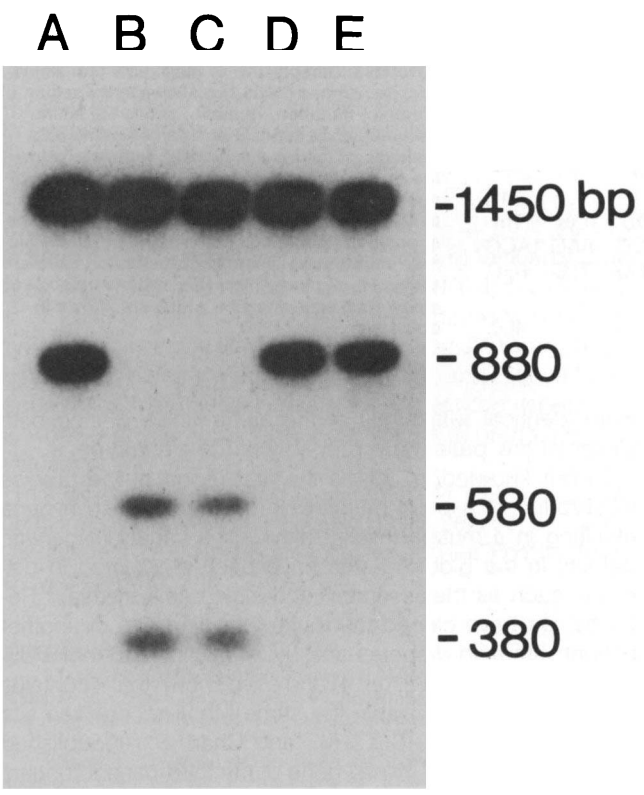

FIGURE 1. Analysis of restriction endonuclease MboII digested DNA fragments from five clones containing the human insulin gene of patient R.C. Aliquots of 0.5 μg DNA isolated from the five clones were digested with MboII and fractionated on a 2% agarose gel. The DNA fragments were then transferred to nitrocellulose and hybridized with a cloned human insulin cDNA probe as described earlier.[3] The hybridizing bands were visualized by autoradiography. Their positions are marked in base pairs.

three hybridizing bands of 1450, 580, and 380 base pairs, respectively, while the other three (A, D, and E) produced only two hybridizing bands of 1450 and 880 base pairs. This result was consistent with our earlier findings on Southern blot analysis of leukocyte DNA from this patient;[3] thus, the first two clones represent the "normal" allele and the latter three represent the "abnormal" allele. One clonal isolate of each allele was taken for further analysis.

Restriction mapping of the DNA of these two isolates with restriction endonucleases XhoI, SacI, BglI, BglII, and PvuII showed that these two alleles differ in length by 450 base pairs between the BglI and BglII sites at the 5′ flanking region (Figure 2A). This restriction fragment length difference is consistent with the presence of an insertion or deletion within a tandemly repeating sequence of variable length in that region.[7-10] A large DNA insertion/deletion sequence (1600–2300 base pairs) has been reported to be associated with the occurrence of non-insulin-dependent (type II) diabetes mellitus.[8,9] However, the insertion/deletion sequences in both alleles of this patient belong to the smaller class (0–500 base pairs). Furthermore, the relatively small difference in length of the insertion/deletion sequences in these two alleles does not seem to affect gene expression significantly, since analysis of the patient's pancreatic insulin by HPLC showed that the ratio of the mutant to normal forms was 57:43, indicating that the two genes are expressed more or less equally.[4]

The nucleotide sequences of both alleles between the putative cap site and 180 base pairs after the termination codon (except the second intervening sequence) were determined using the strategy shown in Figure 2B. The nucleotide sequence in the coding region of the normal allele agrees completely with those already published.[11,12] However, there was a C (cytidylate) to G (guanylate) transversion

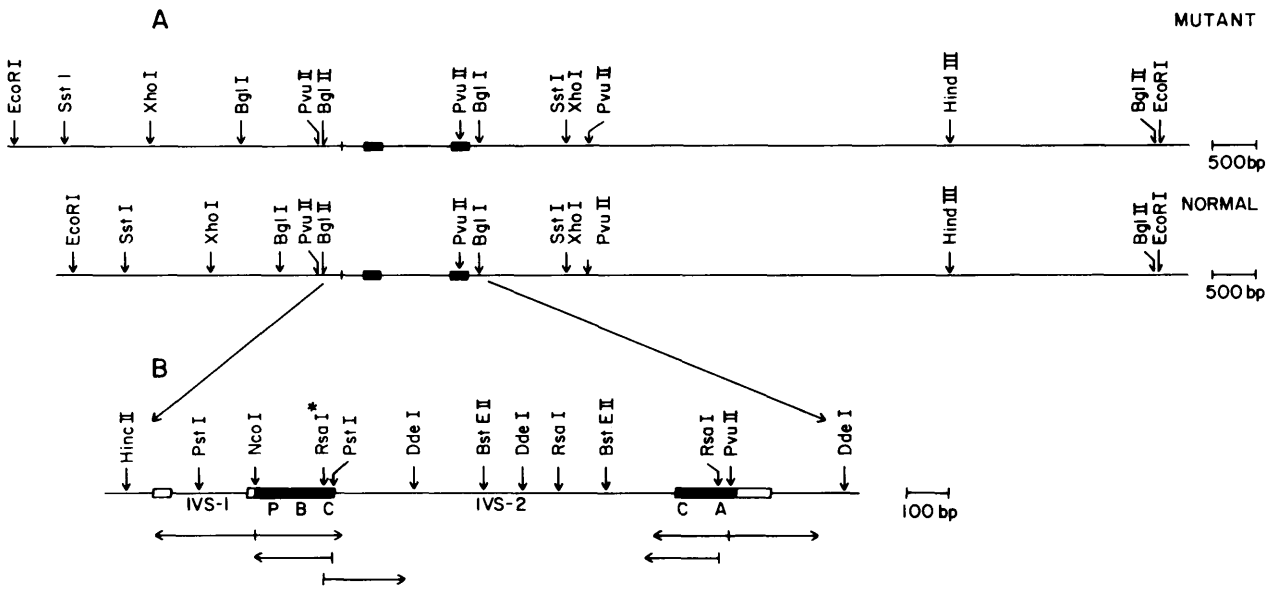

FIGURE 2. Restriction endonuclease map and strategy for nucleotide sequence analysis of the human insulin gene. (A) Restriction endonuclease map of both the normal and mutant alleles of the patient's insulin gene. Only the relevant restriction endonuclease sites within the 14-kilobase EcoRI insert are shown. The boxes enclose the coding sequences of the human insulin gene. (B) Strategy for nucleotide sequence analysis of the gene. Only the restriction endonuclease sites used in DNA sequence analysis are shown. The RsaI site marked by an asterisk (*) appears only in the mutant allele. The filled and open boxes represent coding and untranslated sequences, respectively. IVS-1 and IVS-2 denote the positions of the two intervening sequences while P, B, C, and A indicate the coding regions for the prepeptide, B-chain, C-peptide, and A-chain of insulin, respectively. The horizontal arrows indicate the direction and extent of each sequence analysis.

Insulin B-chain

	20										30
Normal	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr
	GGG	GAA	CGA	GGC	TTC	TTC	TAC	ACA	CCC	AAG	ACC
	CCC	CTT	GCT	CCG	AAG	AAG	ATG	TGT	GGG	TTC	TGG
	'					MboII					'
	370										400
	20										30
Mutant	Gly	Glu	Arg	Gly	Phe	**Leu**	Tyr	Thr	Pro	Lys	Thr
	GGG	GAA	CGA	GGC	TTC	TT**G**	TAC	ACA	CCC	AAG	ACC
	CCC	CTT	GCT	CCG	AAG	AAC	ATG	TGT	GGG	TTC	TGG
	'	.					RsaI				'
	370										400

FIGURE 3. Comparison of nucleotide and amino acid sequences of the two alleles in the region of the point mutation. Numbers above the amino acid sequence indicate position of amino acid residues of the insulin B-chain. Numbers below the nucleotide sequence indicate the position of nucleotides from the putative cap site. A single C to G transversion at the codon for phenylalanine at position 25 resulted in a leucine for phenylalanine substitution, the loss of restriction endonuclease MboII recognition site, and the creation of a new RsaI cleavage site, which are shown in bold type.

at nucleotide 385 in the mutant allele (Figures 3 and 4), which predicts a leucine for phenylalanine substitution at position 25 of the insulin B-chain as proposed by Shoelson et al.[4] This point mutation also abolishes the recognition site for restriction endonuclease MboII (TCTTC) as demonstrated by restriction analysis with MboII (Figure 1 and ref. 3). On the other hand, the point mutation creates a new RsaI site (GTAC), which has also been confirmed by digestion with restriction endonuclease RsaI (Figure 2B). No other mutations were observed.

Four single base substitutions have been observed in the two allelic human insulin genes that have been studied previously;[11,12] two are located in the 3′ untranslated region, and the other two occur within the two intervening sequences. However, these base substitutions do not seem likely to affect either gene expression or insulin molecular structure. Although the site of substitution within the second intervening sequence (nucleotide 1045) was not determined for the two alleles of this patient, the other three bases in both alleles were identical with those of the alpha-type.[12] Hence both alleles of this patient are probably of the alpha-type.

To our knowledge, this is the first report of the precise localization of a point mutation in the human insulin gene resulting in a mutant insulin, although a variety of genetic defects in the globin genes have been pinpointed in diseases such as thalassemia[13] and sickle cell anemia.[14] Recently, we have cloned the mutant insulin gene of another patient with mild diabetes and hyperinsulinemia from a different family (patient 2, ref. 4) and have shown that it contains a different mutation within the same MboII recognition site (Haneda, M., Kwok, S. C. M., and Chan, S., unpublished results). The mutant insulin gene of the third patient (patient 3, ref. 4) has not yet been cloned; however, restriction analysis with endonuclease MboII on the DNA from this patient showed that the mutation does not occur within the same MboII recognition site.[4] Besides these structurally abnormal insulins, structurally abnormal proinsulins also have been reported in patients with hyperproinsulinemia.[15,16] Recom-

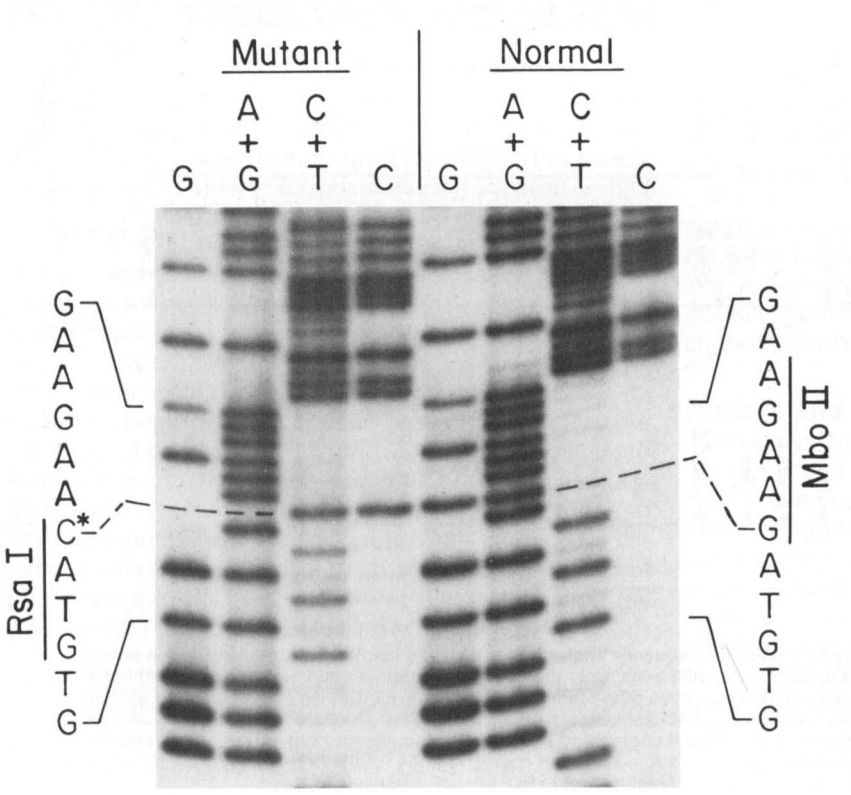

FIGURE 4. Autoradiograph of portions of the sequencing gel showing the partial nucleotide sequence of the anti-coding strand of both normal and mutant alleles in the area of the point mutation. DNA was cleaved with restriction endonuclease PstI, labeled at the 5′ end and cleaved again with a second enzyme NcoI to produce single-end labeled fragments for nucleotide sequence analysis by the procedure of Maxam and Gilbert.[6] The single base transversion is indicated by an asterisk (*). For those unfamiliar with reading nucleotide ladders, only one nucleotide is present at each position, the sequence starting at the bottom and reading upward. If a band is present in the A + G lane and no band is seen in the G lane, the nucleotide is A. If the band is present in both G and A + G, the nucleotide is G. The same procedure holds for C + T and C. The scale on either side refers to the sequence between the bracketing lines marked by the initial and terminal G of the sequence. The recognition sites for restriction endonucleases MboII and RsaI are underlined.

binant DNA technology clearly provides a powerful tool for characterizing these structurally abnormal insulins or proinsulins directly at the genetic level. The results will undoubtedly lead to a better understanding of the relative importance of mutations in the insulin gene in relation to the incidence of various types of diabetes mellitus.

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