the abnormal insulin described above which confirms the assignment of this defect to the Phe-Phe pair at positions 24 and 25 in the B chain.

MATERIALS AND METHODS

DNA was prepared from the leukocytes collected from 50-100 ml heparinized blood by digestion with 100 μg/ml proteinase K (EM Biochemical) at 50°C for 3 hr. in the presence 0.5% SDS (sodium dodecyl sulfate), followed by phenol extraction (3 times) and dialysis against 10mM TrisHCl, pH 7.5, 1mM EDTA. The dialyzed DNA was further purified by digestion with 100 μg/ml ribonuclease A (PL-Biochemicals) at 37°C for 2 hr. and then with proteinase A as described above, followed by phenol extraction (twice) and ethanol precipitation. The DNA precipitate was redissolved in 5 ml of 10mM TrisHCl, pH 7.5, 1mM EDTA and dialyzed against 1mM TrisHCl, pH 7.5, 0.1mM EDTA. DNA samples (30 μg) were digested with 2 fold excess restriction endonuclease MboII (New England Biolabs) at 37°C overnight. The DNA fragments were then separated on a 2% agarose gel and transferred to nitrocellulose by the method of Southern (8). The nitrocellulose filter was first incubated at 65°C for 24 hr. with 10 ml of 3X SSC (450mM NaCl, 45mM sodium citrate, pH 7.0), 10X Denhardt's solution (9), 50mM sodium phosphate, pH 6.5, 200 μg/ml alkali denatured salmon testis DNA (Sigma), 10 μg/ml poly (A), 10 μg/ml poly (C) (PL-Biochemicals) and 0.1% SDS. The filter was then hybridized at 65°C for 3 days with cloned human insulin cDNA (10), ^{32}P-labeled by nick translation (11) (2-3 x 10^6 CPM/filter) in 5 ml of the above solution. After hybridization, the filter was first washed twice with 2x SSC, 0.1% SDS at room temperature for 10 min. each, then six times with 0.1X, SSC, 0.1% SDS at 65°C for 30 min. each and was finally rinsed rapidly with 0.1X SSC at room temperature. The filter was blotted dry and exposed to X-ray film at -70°C for 7 days in the presence of an intensifying screen (Dupont).

RESULTS AND DISCUSSION

The rationale for this study is shown in Fig. 1. The DNA sequence of the codons for the phenylalanine residues at positions 24 and 25 of the insulin B chain (TTC-TTC) contains the recognition site for restriction endonuclease MboII (TCTTC). Examination of the known nucleotide sequence of the human insulin gene (6,7), reveals two additional MboII restriction sites; one located 336 base pairs upstream and the other 555 base pairs downstream from the site corresponding to Phe^{B-24}-Phe^{B-25}. Other MboII sites are presumably present beyond the 3'-untranslated region, but do not lie within the known nucleotide sequence (6,7). Based on the above data, digestion of genomic DNA containing a normal insulin gene with MboII should generate two insulin-related DNA fragments of 336 and 555 base pairs and a third fragment of undetermined size. A mutation in the DNA for either phenylalanine codon resulting in the substitution of leucine at position 24 or 25 of insulin B chain would destroy the MboII site, with the single exception of the transition TTCTTC→CTCTTC. Thus, 11 of the 12 possible mutations which could give rise to leucine coding in this region should result in the

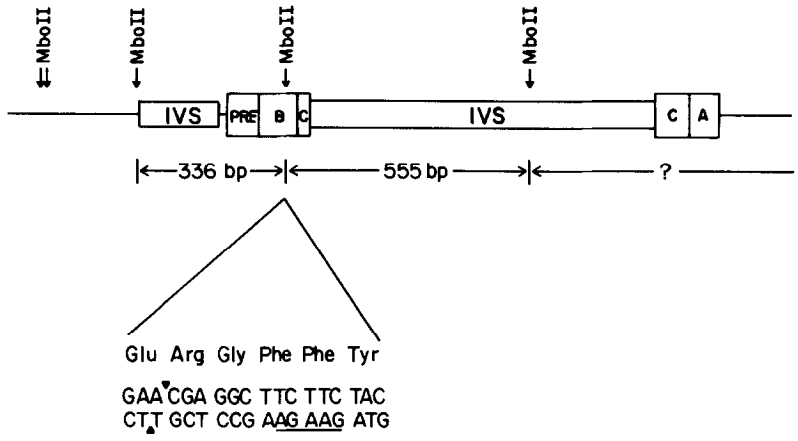

Figure 1. Schematic representation of the human insulin gene structure showing sites of MboII cleavage in relation to coding (Pre,B,C,A) and intervening sequences (IVS) as predicted from the nucleotide sequence studies of Bell, et al. (6) and Ullrich, et al. (7).

generation of an insulin-related DNA fragment of 891 base pairs (the sum of the 336 and 555 base pair fragments) upon digestion with Mbo II. Since the abnormal insulin constitutes only 60% of the total insulin in this patient (3) the two normal insulin-related DNA fragments (336 and 555 base pairs) should also be detected.

As expected, DNA from a normal subject digested with MboII gave three DNA bands of 1600, 580, and 400 base pairs which hybridized with the human insulin cDNA probe (Fig. 2, lane A). The 580 and 400 base pair bands agreed closely with the expected sizes based on the known nucleotide sequence. The 400 base pair band was quite faint possibly due to selective loss on nitrocellulose during the washing procedure. Although the size of the 1600 base pair band could not be predicted from the known nucleotide sequence, it clearly contained sequences related to the 3'-end of the insulin mRNA since it also hybridized strongly with a human insulin cDNA probe lacking the B chain sequence (10).

In contrast, after digestion with restriction endonuclease MboII and hybridization the DNA from the patient revealed a band of 980 base pairs in addition to the three bands observed with the normal subject's DNA (Fig. 2, lane B). The 980 base pair fragment had the size expected for a fragment containing the 580 and 400 base pair

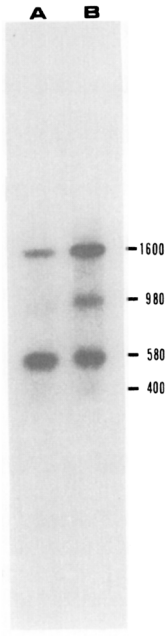

Figure 2. Analysis of restriction endonuclease MboII digested DNA fragments from a normal subject (lane A) and a diabetic patient with an abnormal insulin (lane B). DNA samples were digested with MboII and fractionated on a 2% agarose gel. The DNA fragments were then transfered to nitrocellulose and hybridized with a cloned human insulin cDNA probe as described in Methods. The hybridizing bands were visualized by autoradiography. Their positions are marked in base pairs.

fragments. Digestion of the DNA with a 2-fold excess of enzyme did not cleave this 980 base pair fragment indicating that this result was not due to incomplete enzymatic digestion. Thus we conclude that the MboII site is indeed absent in a portion of the patient's DNA due to a mutation in the region coding for either Phe^{B-24} or Phe^{B-25}. These results strongly support the conclusions of Tager, et al. (3) that a leucine for phenylalanine substitution at position 24 or 25 of the insulin B chain accounts for the interesting clinical and laboratory findings in this patient. Our results are also consistent with the probable heterozygosity of this defect.

Based on the similar antagonistic activities of the abnormal insulin isolated from the patient (2) and a semisynthetic Leu $^{B-24}$-insulin analog (12), Tager, et al. have further postulated that the leucine for phenylalanine substitution has occurred at position 24 of the insulin B chain. If this is the case and a single base change has occurred,

the codon for the amino acid at position B-24 in the messenger RNA for the abnormal insulin would most likely be UUA or UUG instead of UUC. As noted above, mutation resulting in CUC is unlikely because this would not alter the MboII site. The exact identification of this point mutation must await the cloning and nucleotide sequence analysis of the 980 base pair fragment which is currently in progress in our laboratory. These results indicate that restriction mapping of genes can be a useful diagnostic tool for studies of genetic disorders such as diabetes due to structural abnormalities in insulin or its precursors.

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