A Novel Point Mutation in the Human Insulin Gene Giving Rise to Hyperproinsulinemia (Proinsulin Kyoto)

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Abstract

We have identified a 65-yr-old nonobese Japanese man with diabetes mellitus, fasting hyperinsulinemia (150–300 pM), and a reduced fasting C-peptide/insulin molar ratio of 2.5–3.0. Fasting hyperinsulinemia was also found in his son and daughter. Analysis of insulin isolated from the serum of the proband and his son by reverse-phase high performance liquid chromatography revealed a minor peak coeluting with human insulin and a major peak of proinsulin-like materials. The insulin gene of the patient was amplified by the polymerase chain reaction and the products were sequenced. A novel point mutation was identified in which guanine was replaced by thymine. The substitution gives rise to a new HindIII recognition site and results in the amino acid replacement of leucine for arginine at position 65. These results indicate that the amino-acid replacement prevents recognition of the C-peptide–A chain dibasic protease and results in an elevation of proinsulin-like materials in the circulation. Furthermore, in this family the proinsulin-like materials is due to a biosynthetic defect, inherited as an autosomal dominant trait. Rapid detection of this mutation can be accomplished by HindIII restriction enzyme mapping of polymerase chain reaction–generated DNA, which enables us to facilitate the diagnosis and screening. (*J. Clin. Invest.* 1992. 89:1902–1907.) Key words: clinical study • high performance liquid chromatography • polymerase chain reaction amplification • restriction site polymorphism

Introduction

Proinsulin, a large molecular weight polypeptide precursor consisting of 86 amino acids, is cleaved into insulin and connecting peptide (C-peptide), which are released into the blood in equimolar amounts (1). In normal subjects, only small amounts of proinsulin enter the circulation, and the concentration of proinsulin is generally less than 20% of that of insulin (2–4), although proinsulin concentrations are frequently elevated in the patients with diabetes (4, 5). It has recently been

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reported, however, that hyperproinsulinemia or hyperinsulinemia may occur in association with structural abnormalities in the proinsulin or insulin molecule due to single nucleotide substitutions in the insulin gene. 10 families have been reported in which some members have single point mutations in the insulin gene that result in amino acid substitutions within the proinsulin molecule. Among them, six families have mutations in which the abnormal insulin has essentially normal immunoreactivity but is biologically defective; in three Japanese families A3-Val was replaced with Leu (insulin Wakayama [6–8]); in two independent families in the United States and Canada B24-Phe was replaced with Ser (insulin Los Angeles [9–11]); and in a single family in the United States B25-Phe was replaced with Leu (insulin Chicago [12, 13]). Few additional families have been identified in which the mutations are associated with hyperproinsulinemia. One of these has a substitution of B10-His with Asp (proinsulin Providence [14, 15]), resulting in a proinsulin that demonstrates markedly altered subcellular sorting behavior (16, 17). An additional point mutation in three families has been identified that leads to replacement of Arg-65 by His (proinsulin Tokyo), and prevents recognition of the C-peptide–A chain dibasic cleavage site (Lys-Arg) by the processing protease in the pancreatic β cells (18–20). The present report describes in detail the clinical and laboratory characterization of another diabetic patient who appears to have a novel mutation in the insulin gene.

Methods

Case report. The patient is a 65-yr-old nonobese Japanese man who was found to have asymptomatic glucosuria on routine urine examination in 1978. An oral glucose tolerance test (OGTT)[1] revealed impaired glucose tolerance and hyperinsulinemia (fasting plasma glucose level of 4.6 mM and serum immunoreactive insulin [IRI] level of 150 pM). In 1985, he developed polyuria and polydipsia. Physical examination was normal, except for background diabetic retinopathy. Routine laboratory examinations revealed mild hyperglycemia (fasting plasma glucose level of 7.4 mM) and high level of fasting IRI (290 pM). Since that time, he has been treated with diet therapy without medications.

Clinical studies. In January 1988, the patient entered the hospital for further clinical and laboratory studies. Serum samples were collected to determine the levels of plasma glucose, IRI, and C-peptide immunoreactivity (CPR) during OGTT at baseline and 30, 60, 90, and 120 min after oral administration of 75 g of glucose. For an insulin tolerance test, human actrapid insulin (Novo Nordisk, Copenhagen, Denmark; 0.1 U/kg) was injected intravenously after an overnight fast. Counter-insulin hormones, insulin antibodies, and insulin receptor an-

tibodies were also determined. Levels of fasting glucose and IRI were also measured in his son and daughter.

Characterization of circulating insulin. A stimulated serum sample was obtained from the patient 60 min after oral administration of 75 g glucose. 3 ml of serum was fractionated on a Biogel P-30 (Bio-Rad Laboratories, Richmond, CA) column, 1 by 60 cm, equilibrated in borate buffer pH8.6 containing 0.4% BSA. The insulin immunoreactivity in each column fraction (0.5 ml) was determined by radioimmunoassay.

Analysis of serum insulin-like immunoreactivity by reverse-phase HPLC. To further characterize the nature of circulating insulin and proinsulin in this patient, serum insulin and proinsulin immunoreactivity were extracted by immunoaffinity chromatography with a nonspecific guinea pig anti-insulin immunoglobulin, coupled to agarose beads (Affi-Gel 10; Bio-Rad Laboratories) using modifications of previously described methods (21). Proinsulin and the proinsulin conversion products demonstrate 100% cross-reactivity with these antibodies when compared with insulin. Separation of insulin was performed by reverse-phase HPLC, using a Series 4 liquid chromatograph, ISS-100 column oven, and LC-100 recorder/integrator (Perkin-Elmer Corp., Norwalk, CT). Reconstituted immunopurified insulin and proinsulin (165 μl) were injected onto an Ultrasphere Ion pair C-18 column, 4.6 mm $\times$ 25 cm, 5 μm particle size (Beckman Instruments, Berkeley, CA) at an elution rate of 1 ml/min and temperature of 45°C. Each HPLC run began by equilibrating the column for 15 min with elution buffer I (20% acetonitrile and 80% [0.012 M triethylamine, 0.1 M phosphoric acid, and 0.05 M NaClO$_4$ {TEAP}], pH 3.0) at a concentration of 67% and elution buffer II (50% acetonitrile and 50% TEAP, pH 3.0) at 33%. For peptide separation, a linear gradient was produced by increasing the overall concentration of elution buffer II to 46% over the course of 90 min. The level of immunoreactivity in column fractions that coeluted with the insulin standard was measured in the insulin radioimmunoassay. Immunoreactivity in column fractions that coeluted with the insulin standards of intact proinsulin and the proinsulin conversion intermediates were measured using a proinsulin ELISA in which the two major proinsulin conversion products (des 31,32 and 64,65 proinsulin) demonstrate 100% cross-reactivity in the assay in relation to proinsulin.

Insulin gene characterization. Genomic DNA prepared from peripheral leukocytes of the proband and his family were used as a template for the polymerase chain reaction (PCR) (22). Oligonucleotide primer pairs used to PCR amplify exon 2 of the insulin gene were nt 484 to 502 and complementary to nt 689 to 708. For exon 3 of the insulin gene, a set of oligonucleotides used were nt 1435 to 1454 and complementary to nt 1682 to 1700, as described by Bell et al. (23). 1 μg of genomic DNA and 0.1 nmol of each oligoprimer were added to 100 μl of a solution containing the Cetus buffer, 50 μM each dNTP, and 2.5 U of Taq polymerase (Perkin-Elmer Corp.). The mixture was overlaid with mineral oil and placed at 95°C for 3 min before beginning the first cycle. The PCR was carried out for 28 cycles under the following conditions: denaturation for 1 min at 95°C; annealing for 1 min at 58°C; and extension for 0.7 min at 72°C. After amplification, the products were extracted with chloroform, precipitated with ethanol, resuspended with water, electrophoresed through a 1.5% agarose gel, and visualized with ethidium bromide. The bands corresponding to the exon 2 and exon 3 of the insulin gene were cut out from the gel, blunt ended with Klenow fragment, kinased with T4 polynucleotide kinase, and then subcloned into the SmaI-digested pUC13. The ligated products were then sequenced by the dideoxy chain termination method (24), using universal primers and sequence-specific primers and Sequenase Ver 2.0 (United States Biochemical Corp., Cleveland, OH).

Southern blot analysis. 10 μg of genomic DNA of controls, the proband, his son, daughter, and cousin were digested with HindIII, electrophoresed through a 0.7% agarose gel, transferred to a nylon filter, and baked in a vacuum oven at 80°C for 2 h. The blot was prehybridized at 42°C and hybridized overnight at 42°C with the ^{32}P-labeled BamHI fragment of phins 96 (a kind gift from Dr. G. I. Bell, The University of Chicago) in a solution as described previously (25).
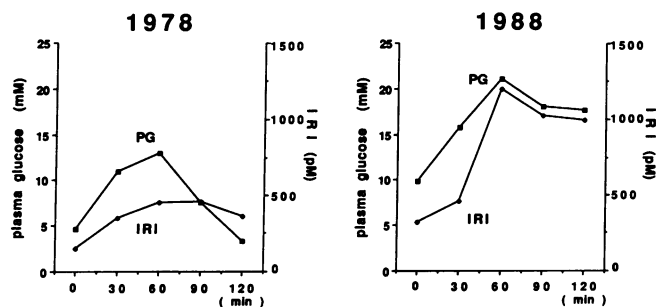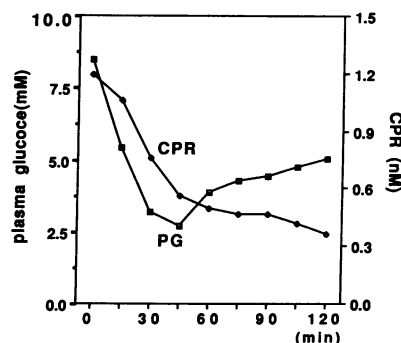

Figure 1. Oral glucose tolerance test profiles. 50 g glucose in *1978* and 75 g glucose in *1988* were used. Note that he became diabetic pattern in *1988*. PG indicates plasma glucose (mM) and IRI indicates insulin immunoreactivity (pM).

The blot was washed twice at room temperature for 30 min, and then at 55°C for 30 min in 0.1× standard saline citrate (SSC), 0.1% SDS before autoradiography.

Results

Clinical studies. The oral glucose tolerance test revealed that the patient had become diabetic and hyperglycemia occurred despite high levels of IRI (Fig. 1). Both fasting (319 pM) and glucose-stimulated (a peak value of 1,200 pM at 60 min) IRI levels were high, and the fasting plasma glucose level was 9.8 mM, which increased to 21.1 mM at 60 min and remained elevated at 120 min. In contrast to insulin, the plasma CPR levels were relatively normal; fasting and glucose-stimulated CPR levels were 1.0 and 4.7 nM, respectively. The resultant CPR/IRI molar ratio was relatively low (2.5–3.1 for the patient; > 4.0 for healthy control). Proband's levels of plasma glucagon (88 ng/liter), growth hormone (0.1 μg/liter), thyroid hormone (T3 1.4 nM, T4 103 nM), and urinary 17-OHCS (8.0–18.0 μmol/d) were all within normal limits. Moreover, the patient's serum did not contain insulin antibodies or insulin receptor antibodies. To assess insulin sensitivity in vivo, exogenous insulin was injected intravenously. In spite of the high endogenous IRI levels, the patient responded normally to exogenous insulin (Fig. 2).

Family studies. The results of OGTT profiles of his healthy son and daughter and a pedigree are summarized in Fig. 3. Examination of the patient's family by an OGTT revealed that his son and daughter had marked hyperinsulinemia in spite of the normal glucose tolerance. The results indicate that the abnormality in this family is inherited by an autosomal trait.

Figure 2. Insulin tolerance test profile. The response of plasma glucose to an intravenous bolus injection of 0.1 U/kg of human insulin is shown. The basal glucose level fell from 8.5 mM to a nadir of 2.7 mM 45 min after the insulin infusion.
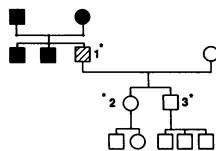
Figure 3. Pedigree and 75 g oGTT profiles for his children. Numbered subjects were available for screening. (1) Proband (2) Daughter (3) Son. *Fasting hyperinsulinemia and the diagonal line signifies a diabetic subject.

Subject	Age /Sex		0'	30'	60'	90'	120' (min)
2	41 F	PG	4.8	6.9	7.4	6.5	5.4 (mM)
		IRI	420	888	1326	1416	1158 (pM)
3	36 M	PG	4.3	7.3	8.8	8.2	5.3 (mM)
		IRI	228	450	648	990	978 (pM)

Gel filtration study. Two well-separated peaks of immunoreactivity were found (Fig. 4). By use of standards of human proinsulin and insulin, the first peak and second peak correspond to proinsulin and insulin, respectively. Thus, the serum of the patient showed a markedly increased peak of proinsulin-like materials (PLM), with a smaller insulin peak. The PLM peak in the patient constituted 87% of total IRI eluted from the column.

HPLC study. Insulin of the proband and his son purified from peripheral venous blood was applied to an HPLC column. The results are shown in Fig. 5. The elution profiles of insulins demonstrated two peaks of immunoreactive insulins. One small peak (~ 5% of total IRI) appeared at the position of normal human insulin. Another large peak (~ 95% of total IRI) appeared in a position different from normal human proinsulin or the normal proinsulin conversion products, and was assumed to be due to a structurally abnormal proinsulin.

Insulin gene characterization. To determine the sequence of the patient's insulin gene, genomic DNA from the peripheral blood leukocytes was PCR amplified. Resultant products of exon 2 and exon 3 of the insulin gene were subcloned into pUC13 and nucleotide sequencing was performed. Five of the recombinant pUC13 containing exon 2 showed an entirely normal sequence. On the other hand, two of the recombinant pUC13 containing exon 3 were normal, and the others showed a G → T point mutation that corresponds to the second codon position of amino acid residue 65 of Arg (Fig. 6). This site also corresponds to a dibasic site, Lys-Arg at a junction of C-peptide and the A chain, and the point mutation creates a new CTT codon encoding the amino acid leucine (Fig. 7). Moreover, the resultant nucleotide sequences around the point mutation created a HindIII recognition site (AAGCGT → AAGCTT).
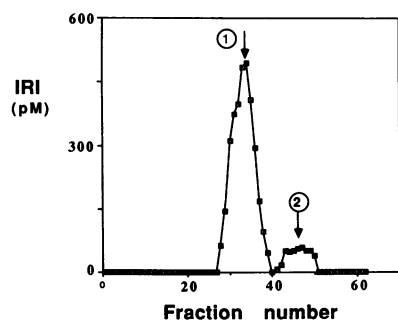

Figure 4. Biogel P-30 column chromatography of 3 ml of serum from the proband. Insulin immunoreactivity was measured in each column fraction using a double antibody method and a human insulin standard. The results are expressed as pM per 100 μl of each 0.5 ml fraction. Human proinsulin (1) and insulin standards (2) eluted from the column in the positions shown by the arrows.
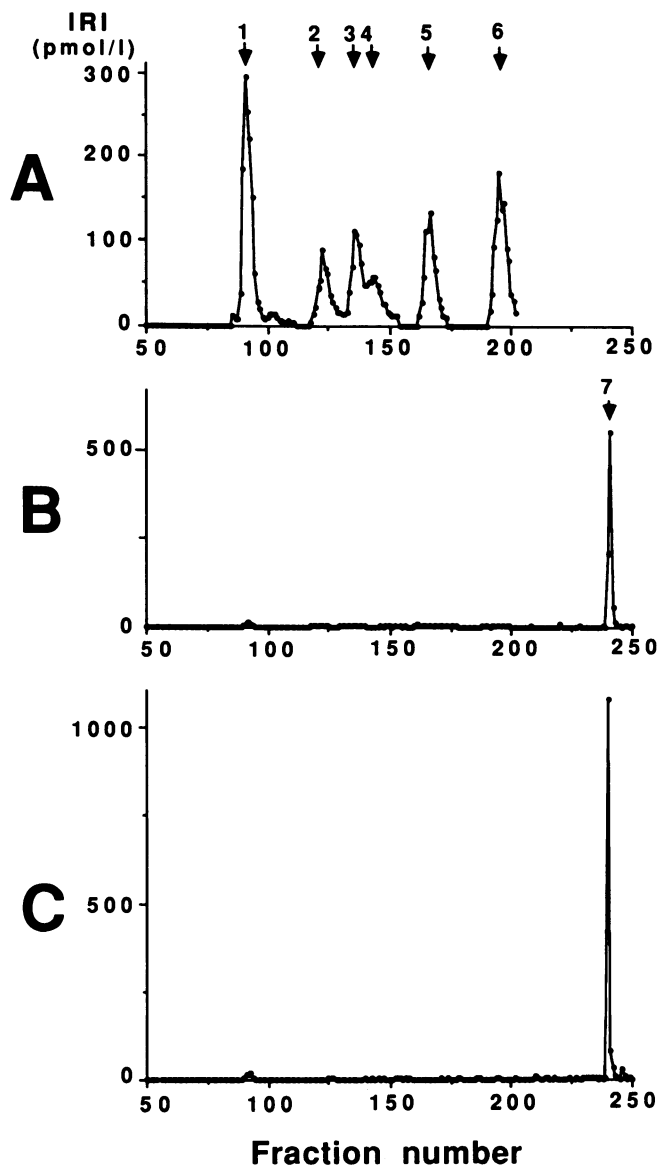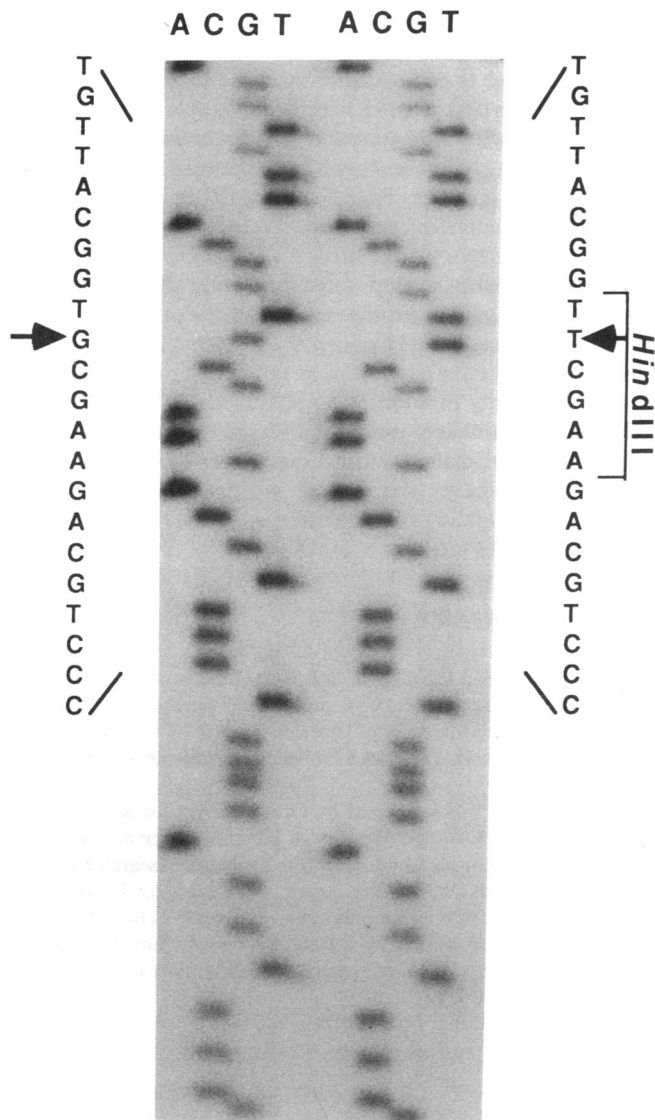

Figure 5. Separation of insulin immunoreactivities in the proband and son by reverse-phase HPLC. Samples were collected 60 min after 75 g of oral glucose. (A) Standard human insulin; (B) IRI from the proband; (C) IRI from the son. Each number of the peak indicates as follows: (1) human insulin; (2) ACw Bacics 32,33 split human proinsulin; (3) AC des 31,32 human proinsulin; (4) BCw Bacics 65,66 split human insulin; (5) BC des 64,65 human proinsulin; (6) human proinsulin; and (7) mutant proinsulin. HPLC column and the procedures were described in Methods.

Southern blots of HindIII-digested genomic DNA from blood of four family members and two controls were probed with a 1.7-kb human insulin gene fragment from phins96 including the whole insulin structural gene (Fig. 8). An ~ 11-kb fragment was found in all six persons examined. In addition to this fragment, 5.8- and 5.6-kb fragments were observed in the proband, son, and daughter. These results confirm that three members of the family are heterozygous for the mutation that creates HindIII recognition site. Since the maternal cousin did not show hyperinsulinemia his Southern blot was normal as controls.

Figure 6 (sequence gel)

```
      A C G T   A C G T
T \                          / T
G                              G
T                              T
T                              T
A                              A
C                              C
G                              G
G                              G
T                              T
G ←  (arrow)              ← T ] HindIII
C                              C
G                              G
A                              A
A                              A
G                              G
A                              A
C                              C
G                              G
T                              T
C                              C
C /                          \ C
C /                          \ C
```

Normal   Mutant
Allele   Allele

Figure 6. Sequence analysis of exon 3 of the insulin gene. The coding strands of both normal and abnormal allele DNA were sequenced by the dideoxy chain termination method. The direction of 5′→3′ is from the bottom to upside. A portion of the autoradiogram from a 5% polyacrylamide/8 M urea gel is shown. A single base substitution (G→T) is indicated by arrows. The recognition site for HindIII is also indicated.

	C-chain			Junction		A-chain
	Leu	Gln	Lys	Arg	Gly	Ile
Normal allele	CTG	CAG	AAG	CGT	GGC	ATT
	62	63	64	65	66	67
Mutant allele	CTG	CAG	AAG	CTT	GGC	ATT
				Leu		

Figure 7. Comparison of partial nucleotide and amino acid sequence of exon 3 of the insulin gene. The numbers indicate the positions of the amino acid from the amino terminus of the B chain. A single G to T transition at the codon for arginine at position 65 resulted in a leucine substitution and loss of the Lys-Arg pair.
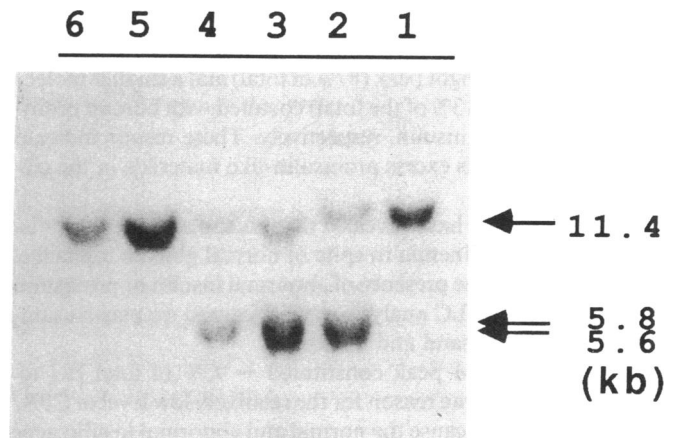
(right column)

Figure 8. Southern blot analysis of HindIII restriction fragments of genomic DNA from members of the family and normal controls. The hybridizing bands are marked in kilobase pairs. (Lane *1* and *6*) Normal controls; (Lane *2*) proband; (Lane *3*) son; (Lane *4*) daughter; (Lane *5*) maternal cousin of the proband.

Discussion

We have identified a non-insulin-dependent diabetic patient who presented with glucose intolerance and hyperinsulinemia. The combination of hyperglycemia and hyperinsulinemia suggested an insulin-resistant state or an abnormal insulin gene product from pancreatic β cells. In general, insulin resistance can be due to circulating insulin antagonists or a cellular defect in insulin action at a receptor or postreceptor site. However, his circulating counter-insulin hormones were not elevated, and neither insulin antibodies nor insulin receptor antibodies were found. He responded normally to exogenously administered insulin. The results indicated that he was not resistant to exogenous insulin. The fasting CPR/IRI molar ratio was relatively low. This was presumably due to the delayed turnover in vivo of the circulating proinsulin-like materials compared with insulin and is compatible with two other cases of hyperproinsu-
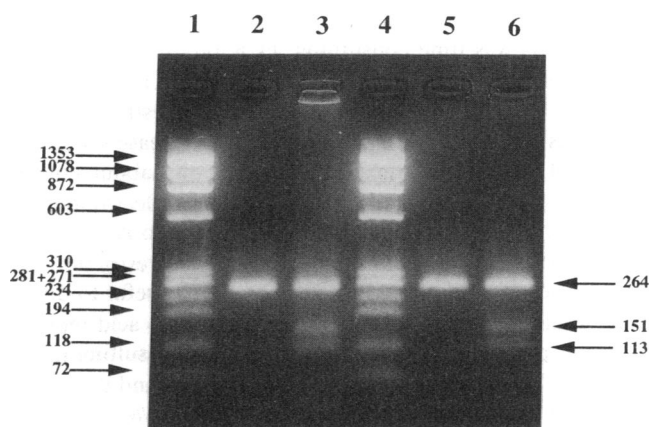

Figure 9. PCR products amplified from genomic DNA including exon 3 of the insulin gene. (Lanes *1* and *4*) Molecular weight marker; (Lanes *2* and *3*) PCR products of exon 3 from the proband; (Lanes *5* and *6*) PCR products of exon 3 from the son. Lanes *3* and *6* indicate the HindIII-digested fragments of lane *2* and lane *5*, respectively. The size of molecular weight marker and fragments (bp) is shown in the left and right, respectively.

linemia. Gel filtration chromatography with Biogel P-30 revealed that the stimulated serum IRI comprised two peaks. The large molecular weight peak (87% of total) and a smaller molecular weight peak (13% of the total) coeluted with human proinsulin and human insulin, respectively. These results indicate that the patient has excess proinsulin-like materials in the circulation.

Family studies have revealed that his son and daughter displayed hyperinsulinemia in spite of normal glucose tolerance. To demonstrate the presence of abnormal insulin or proinsulin in this family, HPLC analysis was performed on plasma samples from the proband and his son.

The large PLM peak constituted ~ 95% of total IRI resulted from the same reason for the relatively low level of CPR/IRI molar ratio because the normal and abnormal insulin gene should be expressed equally. This peak of the family appeared at the more hydrophobic position than normal proinsulin, and did not coincide with human A–C proinsulin (des 31, 32 proinsulin) peak, while previously reported Proinsulin Tokyo or Proinsulin Providence has a peak of A–C proinsulin.

Further evidence in favor of a hypothesis that the PLM in this family resulted from an abnormality in the insulin gene was obtained from the nucleotide sequence of the PCR-amplified insulin gene fragments. A G → T point mutation was found that corresponds to the second codon position of amino acid residue 65 of Arg at a junction of C-peptide and A chain. By contrast, Proinsulin Tokyo also has a single base G to A substitution at the same nucleotide position, resulting in a histidine substitution at Arg-65 (26). Thus, the disappearance of the dibasic pair (Lys-Arg) blocks the maturation of insulin. Because PCR products of the insulin gene were sequenced, an inherent uncertainty in this analysis was the possibility that the coding mutation could be due to a PCR artifact. Attempts to verify the mutation directly in the genomic DNA by the appearance of the predicted HindIII fragments after Southern blot analysis yielded evident results. Moreover, the novel point mutation at the codon for Arg-65 were also confirmed by separate cloning and sequencing of the normal and abnormal insulin alleles.

The prevalence of this novel mutation in the insulin gene among the general population is unknown. Since Southern blot analysis was time consuming, PCR products containing exon 3 were analyzed by HindIII digestion. When the product is divided into three fragments with HindIII digestion, the heterozygous mutation of this newly identified disease should be easily and rapidly confirmed (Fig. 9). Thus, only about 4 or 5 h from the start of PCR to the end of agarose gel electrophoresis is required, while screening of Proinsulin Tokyo is time consuming because it does not create any site of restriction enzyme. These techniques with PCR should be useful to detect other mutant insulin genes giving rise to amino acid replacements at position Phe^{B24} and Phe^{B25}, thereby resulting in the loss of an MboII restriction site. Family studies and their insulin gene analysis revealed that the abnormality was an autosomal dominant trait as other reported mutations in the insulin gene.

It is interesting to consider the reasons why the patient with mutant insulin gene has diabetes. Family studies showed that two children with the mutation were not diabetic. There were no differences in the results of HPLC profiles between those with and without diabetes. The reasons for the differences in their metabolic states are not clear. Although the patient is not resistant to exogenous insulin, it cannot be concluded that he has no resistance to endogenous insulin. Moreover, although he responds to oral glucose with considerable IRI secretory response, normal insulin secretion may not be enough to maintain the plasma glucose level within the normal range because normal insulin is only ~ 5–13% of total IRI. It is possible that other genetic factors may determine whether individuals who secrete mutant forms of insulin develop diabetes. Non-insulin-dependent diabetes develops most frequently after middle age. As Nanjo et al. discussed the reasons (27), expression of diabetes might be explained by other diabetogenic factors, genetic or acquired, affecting a defect in insulin secretion or peripheral insulin sensitivity in addition to possessing a mutant insulin gene. It seems unlikely that mutations in the insulin gene are major causes of diabetes. However, the analysis of development of diabetes by the mutant proinsulin will provide a deeper understanding of the pathogenesis of non-insulin-dependent diabetes mellitus.

Acknowledgments

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