# Normal Coding Sequence of Insulin Gene in Pima Indians and Nauruans, Two Groups With Highest Prevalence of Type II Diabetes

NINA RABEN, FABRIZIO BARBETTI, ALESSANDRO CAMA, MAXINE A. LESNIAK, STEPHEN LILLIOJA, PAUL ZIMMET, SUE W. SERJEANTSON, SIMEON I. TAYLOR, AND JESSE ROTH

**The nucleotide sequence of the insulin gene was determined in American Pima Indians and Micronesian Nauruans, two populations in whom the prevalence of non-insulin-dependent (type II) diabetes mellitus is the highest in the world. The insulin gene was amplified by the polymerase chain reaction to generate single-stranded DNA suitable for direct sequencing. The nucleotide sequences of the coding and adjacent regions of the insulin gene in six Pima Indians and two Nauruans with type II diabetes were identical to previously published insulin gene sequences of nondiabetic subjects. *Diabetes* 40: 118–22, 1991**

Point mutations in the insulin gene that result in structurally abnormal insulin or proinsulin molecules have been identified or suspected in several unrelated families with impaired glucose tolerance or non-insulin-dependent (type II) diabetes (1–9). In each case, the insulin gene defect was associated with high levels of circulating insulin or proinsulin. The existence of mutations of the insulin gene and the evidence that such mutations can contribute to the development of type II diabetes make this gene a candidate locus for type II diabetes. American Pima Indians and Micronesian Nauruans represent relatively homogeneous ethnic groups with the highest reported prevalence of type II diabetes in the world; >50% of adult Pima Indians and ~30% of Nauruans have diabetes (10,11). It has been shown that patients with type II diabetes and hyperinsulinemia have markedly elevated proinsulin levels in plasma and a higher than normal ratio of proinsulin to insulin (12–16). To assess the potential role of mutations in the insulin gene in causing the increased insulin and proinsulin levels, we determined the sequence of the insulin gene in six Pima Indians and two Nauruans with type II diabetes. With the polymerase chain reaction (PCR) and direct sequencing of amplified DNA fragments, we found that the coding sequence of the insulin gene was normal in all patients examined.

From the Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, Maryland; the Clinical Diabetes and Nutrition Section, Phoenix Epidemiology and Clinical Research Branch, NIDDK, NIH, Phoenix, Arizona; and the Lions-International Diabetes Institute, Royal Southern Memorial Hospital, Victoria, and The John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Address correspondence and reprint requests to Dr. Nina Raben, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 10, Room 8S-243, Bethesda, MD 20892.

Received for publication 8 March 1990 and accepted in revised form 28 August 1990.

## RESEARCH DESIGN AND METHODS

Clinical data of diabetic patients and control subjects are shown in Table 1. For all patients, diabetes was diagnosed according to World Health Organization criteria (fasting plasma glucose >7.8 mM and/or 2-h postload plasma glucose during the glucose tolerance test >11.1 mM; 17).

Body mass index (BMI) was used as a measure of obesity. Patients with type II diabetes from both groups had a BMI >27 kg/m$^2$ and were considered obese. The control subjects (2 Nauruans) were weight matched with diabetic patients.

Five milliliters of plasma was gel filtered on a Sephadex G-50 column (1.5 × 90 cm) that had been equilibrated with 0.05 M phosphate buffer (pH 7.4). Aliquots of the fractions were assayed for immunoreactive insulin (IRI) and C-peptide immunoreactivity. IRI was measured by a standard method with antiserum 619 (purchased from the Dept. of Pharmacology, Indiana Univ., Indianapolis, IN) and human insulin as standard. The insulin antiserum used in the assay reacted with human proinsulin with 36% of the potency (on a molar basis) of human insulin. C-peptide immunoreactivity was measured by the double-antibody method with a C-PEP-DA-PEG-RIA-100 kit (IRE, Fleurus, Belgium). Fractions containing the 9000- and 6000-$M_r$ immunoreactive components were separately pooled, and each component was passed through a Sep-Pak C-18 cartridge that had been prewashed with 1 M acetic acid. The retained proinsulinlike and insulinlike components were then eluted with 1 ml 75% ethanol/0.01 N HCl. The eluates, representing partially purified insulin-related materials, were lyophilized and reconstituted in

TABLE 1
Clinical data of diabetic patients and control subjects

| Phenotype and subject | Age (yr) | Sex | BMI (kg/m²) | Plasma glucose (mM) | | Fasting IRI (pM) | Proinsulin IRI* (%) |
|---|---|---|---|---|---|---|---|
| | | | | Fasting | 2-h postload | | |
| Pima Indians | | | | | | | |
| 3441 | 32 | F | 35.9 | 8.8 | 17.0 | 819 | 36 |
| 4270 | 38 | M | 51.7 | 14.3 | 19.7 | 698 | 38 |
| 5403 | 32 | M | 29.3 | 6.0 | 12.1 | 285 | 43 |
| 3096 | 29 | M | 42.1 | 8.6 | 15.1 | | |
| 3352 | 30 | M | 33.0 | 6.9 | 15.7 | 513 | |
| 3474 | 36 | M | 47.9 | 6.5 | 12.8 | 598 | |
| Nauruans | | | | | | | |
| 1605 | 59 | F | 29.7 | 11.4 | | | |
| 1947 | 62 | F | 34.6 | 19.8 | | 178 | |
| 234† | 30 | F | 44.6 | 4.4 | 3.5 | 256 | |
| 251† | 32 | M | 35.2 | 5.3 | 5.1 | 356 | |

BMI, body mass index. IRI, immunoreactive insulin.
*Insulin immunoreactivity in the 9000-$M_r$ pool divided by the sum of insulin immunoreactivity in both the 9000- and 6000-$M_r$ peaks (see text).
†Nondiabetic Nauruans.

0.01 N HCl for further analysis by reverse-phase high-performance liquid chromatography (HPLC).

Partially purified plasma insulin and proinsulin were processed further on a C-18 ion-pair reverse-phase chromatography column (0.46 × 25 cm, 5-μm particle size, Altex Scientific, Berkeley, CA) according to the method of Robbins et al. (18) utilizing isocratic mixtures of acetonitrile in triethylamine-containing buffer. Fractions (0.5 ml) were collected every 30 s and dried under vacuum; the residues were dissolved in buffer for determination of IRI and proinsulin.

Human DNA was prepared from whole blood as previously described (19). Briefly, 0.5 ml blood was mixed with 0.5 ml lysis buffer (0.32 M sucrose, 1 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, and 1% Triton X-100) and centrifuged at 13,000 × *g* for 20 s. After two washes with the buffer, the pellet was resuspended in 0.5 ml PCR buffer with nonionic detergents (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, and 0.45% Tween 20), and 30 μg proteinase K was added to the buffer immediately before use. The final resuspension of the pellet containing nuclear DNA in a proteinase K–PCR buffer was incubated at 60°C for 1 h and then at 95°C for 10 min to inactivate the protease. The lysate (10–25 μl) equivalent to ~0.5–1.0 μg of genomic DNA was used for enzymatic amplification of the insulin gene via PCR.

Genomic DNA was amplified with 2.5 U of *Taq* I DNA polymerase as described previously (20). The reaction mixtures (100 μl) contained 50 pM each of oligonucleotide primers flanking exon 2 or 3 of the insulin gene (Table 2). Different sets of oligonucleotide primers were used to separately amplify exon 2 (which codes for the signal peptide, B chain, and $NH_2$-terminal portion of the C-peptide) and exon 3 (COOH-terminal portion of the C-peptide and the A chain). With a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT), each sample was subjected to 35 repeated cycles of 90 s at 94°C, 60 s at 55°C, and 120 s at 72°C.

An excess of single-stranded DNA (ssDNA) suitable for direct sequencing was generated with a two-stage PCR protocol. For direct sequencing of the insulin gene, we used 5 sets of primers flanking exons 2 and 3 of the gene (Table 2). To produce ssDNA of the sense and antisense strands, aliquots (0.2–1.0 μl) of the first PCR mixture were subjected to 25 rounds of amplification (under conditions described above) with either upstream- or downstream-nested primers. Aliquots of the reaction mixture were electrophoresed

TABLE 2
Primers for polymerase chain reaction (PCR) amplification of exons 2 and 3 of insulin gene

| Exon | Upstream | Downstream |
|---|---|---|
| 2 | 1A 5′ CAGCTCTGCAGCAGGGAGG | 3A 5′ GGGCAGCAATGGGCGGTTGGCTC |
| | 2 5′ GGCACCTGGCCTTCAGCCTG | 3 5′ CATGCTGGGTGGGAGCGCCA |
| 3 | 5A 5′ [GGG GAATTC]TGCGACCTAGGGCTGGCGGG | 7B 5′ GTGGGGCTGCCTGCAGGCTGCGT |
| | 5 5′ GGCACTGTGTCTCCCTGACTG | 7 5′ CCATCTCTCTCGGTGCAGGA |
| | 6B 5′ GCGCGGCACGTCCTGGCAG | 8A 5′ [AAA GAATTC]CGTGGAGAGAGCTGGGAGG |

Oligonucleotides were synthesized on a Coder 300 (Du Pont-NEN, Wilmington, DE) and purified by reverse-phase chromatography on NENSORB PREP columns (Du Pont-NEN). Sequences in brackets represent *Eco*RI recognition site plus three noncomplementary nucleotides added to facilitate enzyme digestion. For direct sequencing of exon 2, oligonucleotides 1A and 3 were used to generate double-stranded DNA (dsDNA); oligonucleotides 2 or 3A served to generate single-stranded DNA (ssDNA) sense and antisense strands, respectively, and as sequencing primers. For direct sequencing of exon 3, we used primers 5A and 8A to generate dsDNA; primers 5, 6B, or 7 to produce ssDNA; and primers 6B, 7B, and 8A to prime the sequencing reaction. Primers 5A and 8A were also used for PCR amplification when cloning technique was applied.

through a 3% NuSieve (FMC Bioproducts, Rockland, ME) 1% regular agarose gel in 1× TBE (90 mM Tris, 65 mM boric acid, and 2.5 mM EDTA [pH 8.3]) to determine the accumulation of both ssDNA and double-stranded DNA (dsDNA). We used different dilutions of the first PCR product for the second amplification to reach the optimal ratio of ssDNA to dsDNA. The second PCR mixture (90 μl) was diluted in $H_2O$ to 400 μl, applied to a Millipore-30 (Ultrafree-MC membrane), centrifuged at 6400 rpm for 3 min, and washed twice with 300 μl $H_2O$, and the retentate (5–10 μl) was dried by speed-vacuum centrifugation.

For the sequencing reaction, the dried retentate (template) was resuspended in 7 μl $H_2O$ and combined with 2 μl 5× Sequenase buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, and 250 mM NaCl; USB, Cleveland, OH) and 1 μl (50–100 ng) sequencing primer. After annealing at 75°C for 5 min, 1 μl 0.1 M dithiothreitol; 2 μl of a mixture of dGTP, dCTP, and dTTP (1.5 μM each); 1 μl [α-$^{35}$S]dATP (1000 Ci/mmol); and 2 U modified bacteriophage T7 DNA polymerase (Sequenase) were added to the annealed template-primer mixture. The labeling reaction continued for 5 min at room temperature, and aliquots were combined with 2.5 μl dideoxy terminator mixture; after a 5-min incubation at 37°C, 4 μl of stop solution was added, and the samples were electrophoresed in a 6% polyacrylamide/8 M urea gel.

DNA sequencing ladders obtained by the direct-sequencing method were readable for at least 350 nucleotides, starting at the fourth nucleotide from the primer. The best sequencing ladders were obtained when the product of the second PCR contained the least dsDNA. Therefore, in some cases, to reduce the accumulation of dsDNA, we filtered the first PCR product through a Millipore-30 membrane to remove the residual amplification primers before the second PCR, and the number of PCR cycles was then increased to 35–40. In addition, we found that the quality of the sequencing ladders was improved when the second PCR product was subjected to alkaline denaturation before the annealing step.

## RESULTS AND DISCUSSION

**Gel filtration profile of circulating insulin and proinsulin.** Plasma samples from three Pima Indians with type II diabetes, when filtered on Sephadex G-50, yielded two peaks of IRI (Fig. 1). The first peak corresponded to the position of human proinsulin standard, and the second peak corresponded to the peak of human insulin. For each of the three plasmas, 36–43% of total IRI eluted from the column at the position of proinsulin (Table 1). In two healthy control subjects, the value was <10% (data not shown). As expected, C-peptide immunoreactivity was recovered at a position close to that of the proinsulin standard, indicating that the first peak of insulin immunoreactivity was proinsulinlike material, i.e., proinsulin and/or proinsulin intermediates (Fig. 1). Disproportionate elevation of immunoreactive proinsulin to insulin in patients with type II diabetes has been described by several groups (12–16). The reason for the increase in the molar ratio of the proinsulinlike components is not clear. We conducted studies to determine whether the increased levels of proinsulinlike components were due to a structural defect in the proinsulin molecule that decreased conversion of proinsulin to insulin.

**HPLC of circulating insulin and proinsulin.** HPLC analysis of partially purified plasma insulin and proinsulin fractions from the three Pima Indians (subjects 3441, 4270, and 5403) showed that both insulin and proinsulin eluted from the C-18 reverse-phase column at the positions of standard human insulin and proinsulin, respectively (data not shown). It is known that the circulating proinsulinlike material consists of proinsulin and two types of intermediates formed during the normal processing of proinsulin (21,22). In previously described families with hyperproinsulinemia (up to 90% of total IRI), due to a mutation at the Arg-65 codon, the proinsulinlike component in serum was largely or exclusively a two-chain intermediate in which the C-peptide was still attached to the insulin A chain; the link to the B chain appears to have been cleaved normally (9, 18, 23, 24). The only form of proinsulin we found in the diabetic Pima Indians with relatively mod-

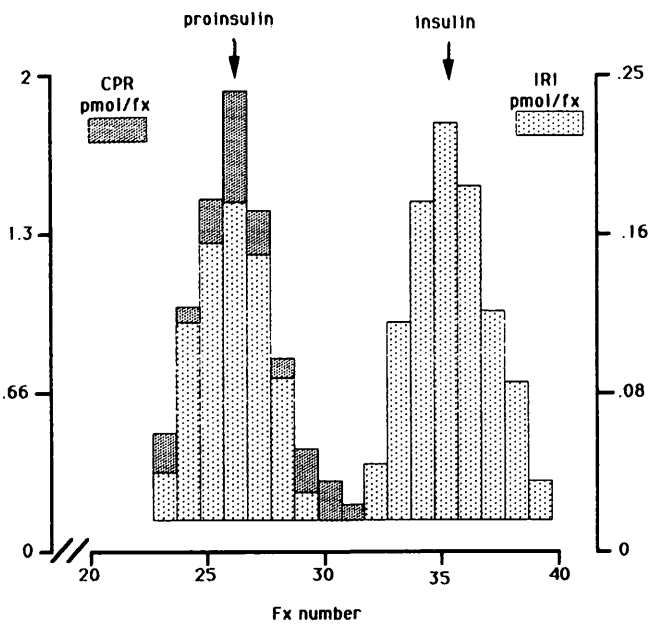


**FIG. 1. Sephadex G-50 gel filtration of plasma from Pima Indian with non-insulin-dependent diabetes mellitus (subject 5403). Plasma (5 ml) was applied to 1.5 × 90-cm column of Sephadex G-50 (fine) that had been equilibrated with 0.05 M phosphate buffer (pH 7.4). Concentrations of insulin immunoreactivity (IRI) and C-peptide immunoreactivity (CPR) in each fraction (fx) are plotted as function of elution volume. Forty-three percent of total insulin immunoreactivity eluted from column at position of proinsulin. This is an underestimate of proportion of proinsulin present in sample, because cross-reactivity of human proinsulin standard with anti-pork insulin antibody used in our assay is 36% on molar basis. Thus, it is likely that concentration of total immunoreactive insulin (including insulin- and proinsulin-related species) may actually be twofold or more higher, and proinsulin-related components may represent up to two-thirds of total insulin immunoreactivity.**

TABLE 3
Allelic variation in insulin gene in Pima Indians and Nauruans with NIDDM

| Phenotype | Pima Indians | Nauruans |
|---|---|---|
| α/α | 3441, 5403, 4270, 3474, 3096 | 234,* 1605 |
| β/β | | 251* |
| α/β | 3352 | 1947 |

Numbers are subject sample codes. NIDDM, non-insulin-dependent diabetes mellitus.
*Nondiabetic subject.

erate hyperproinsulinemia corresponded to normal single-chain intact proinsulin; we did not detect intermediate forms in the eluate fractions. However, normal behavior of proinsulin in our HPLC system does not entirely exclude the possibility of an abnormal molecule. In another family with hyperproinsulinemia (proinsulin levels 33–69% of total IRI), due to a point mutation in the insulin B chain, plasma proinsulin-like material appeared to be a single-chain form that coeluted with normal human proinsulin on reverse-phase C-18 columns (25). Also, the possibility of a nucleotide substitution in the insulin gene producing a mutant insulin with mobility identical to that of normal gene product under the conditions of the HPLC cannot be ruled out.

**PCR amplification and sequencing of insulin gene.** To address directly the question of whether there is a mutation in the insulin gene in patients with type II diabetes, we determined the sequence of the insulin gene from six Pima Indians and two Nauruans with type II diabetes. In three of the Pima Indians (subjects 3441, 4270, and 5403), both protein and DNA were analyzed; in the other three Pima Indians (subjects 3096, 3352, and 3474) and the two Nauruans (subjects 1605, and 1947), only DNA was analyzed. In addition, two nondiabetic Nauruans served as control subjects for the DNA analysis.

The nucleotide sequence of the coding region of the insulin gene was determined by direct sequencing in Pima Indians and Nauruans with type II diabetes and was found to be identical to the sequence of two nondiabetic Nauruans and to normal sequences of the human insulin gene reported by Ullrich et al. (26) and Bell et al. (27).

There are two types of allelic variants of the insulin gene in normal individuals, which differ at four positions: two nucleotide differences in the intervening sequences (IVS) 1 and 2 and two in the 3′ untranslated region (3′ UT). The two types of alleles are designated $\alpha$ and $\beta$. Analysis of directly sequenced PCR products showed that the insulin gene from five Pima Indians and one Nauruan with type II diabetes contained nucleotides corresponding to the α-type allele (Table 3). The insulin gene from these patients has an A residue at position 216 in IVS 1 and C residues at positions 1367 and 1380 in the 3′ UT. (The 4th nucleotide characteristic of the α-allele located in the IVS 2 was not determined.) Because both alleles are detected simultaneously by the direct-sequencing method, we concluded that these subjects are homozygous and contain only α-type insulin genes. The frequency of this allele in the general population has not been reported. In one of the Nauruans, we found only β-type insulin genes, i.e., a T residue in the IVS 1 and T and A residues at positions 1367 and 1380, respectively, in the 3′ UT. Two patients, one from each group, were heterozygous for α- and β-type insulin gene alleles. One control Nauruan subject was homozygous for the α-allele and one homozygous for the β-allele.

In all Pima Indians examined, we also determined the nucleotide sequence of the 3′ region flanking the insulin gene. The sequence of this part of the gene was determined either by direct DNA sequence analysis or with *Eco*RI-digested fragments subcloned into plasmid pGEM 4Z (Promega, Madison, WI) with a standard technique. Of the three single-nucleotide differences between the two published sequences, all three corresponded to the sequence of Ullrich et al. (26; Fig. 2). In addition, in Pima Indians, we found two nucleotides (1 T and 1 C) that were not present in the previously reported sequences. All changes found in Pima Indians corresponded to the current sequence obtained from G.I. Bell (Univ. of Chicago, Chicago, IL). Thus, these differences represent a true sequence polymorphism. In any case, the polymorphisms observed in the 3′ region flanking the insulin gene do not alter the structure of the protein.

The finding of a normal nucleotide sequence in the coding region of the insulin gene in Pima Indians with type II diabetes suggests that structural defects in the gene are not responsible for the elevated proinsulin in this population (the level

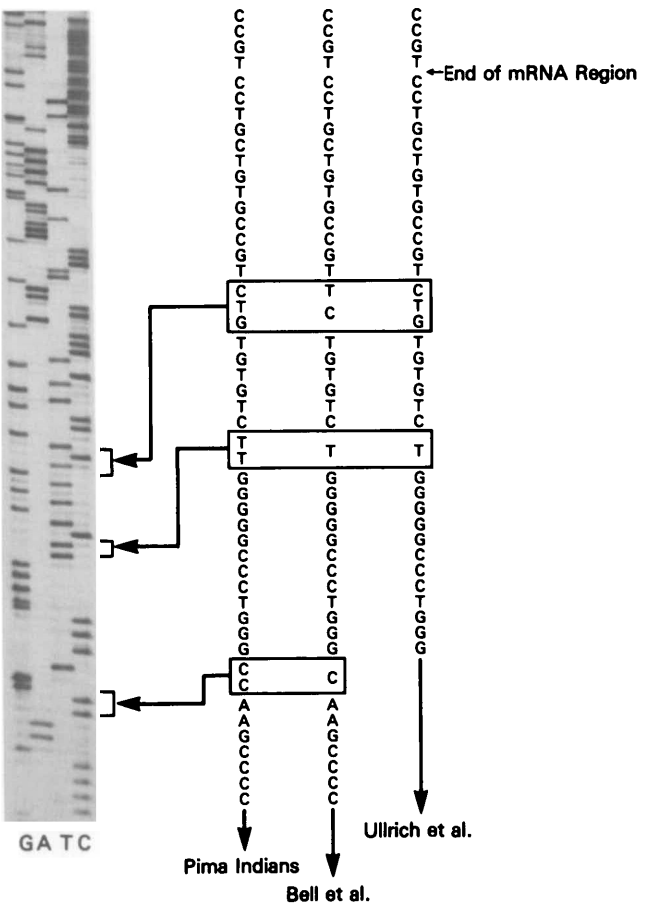


**FIG. 2. Polymorphisms in 3′ untranslated region of insulin gene. Direct-sequence analysis of polymerase chain reaction–amplified DNA by method described in text. Normal human insulin gene sequences are from Ullrich et al. (26) and Bell et al. (27). *Boxes*, regions of nonidentity.**

of plasma proinsulin in Nauruans was not measured). The mechanism of increased serum levels of proinsulin in Pima Indians with type II diabetes is probably like that reported in other patients with type II diabetes and is clearly different from that in patients with familial hyperproinsulinemia. It has been suggested that in patients with type II diabetes, β-cells overstimulated by hyperglycemia release more proinsulin-rich immature granules (13).

Our study provides direct evidence that mutations of the coding region of the insulin gene do not account for type II diabetes in two populations at particularly high risk of developing type II diabetes. Previous evaluations of the insulin gene and the association of this locus with type II diabetes were based on analysis of the polymorphic region 5′ to the insulin gene (restriction-fragment–length polymorphisms), and the results of these studies in different populations were somewhat controversial (28–35). In another approach to estimate the frequency of a previously described B chain Phe-24 and Phe-25 insulin gene mutation, Sanz et al. (36) screened >200 patients with type II diabetes of mixed races and found in one (a 45-yr-old nonobese black male) a nucleotide substitution resulting in a silent mutation; B chain Phe-25 was coded by codon TTT rather than TTC, the codon normally present at this position in the insulin gene (37).

Direct sequencing of the PCR-amplified DNA fragments as described here represents a rapid and informative method for identifying insulin gene mutations and therefore can be applied to screen chosen groups of diabetic subjects for potential defects in this or other candidate genes.

**ACKNOWLEDGMENTS**

This work was partially supported by National Institutes of Health Grant DK-25446 (S.W.S.).

We thank Reviewer I for very helpful comments on the allelic structure, Dr. Domenico Accili for helpful discussions, and Esther Bergman for assistance in preparation of the manuscript.

**REFERENCES**

1. Kwok SCM, Steiner DF, Rubenstein AH, Tager HS: Identification of a point mutation in the human insulin gene giving rise to a structurally abnormal insulin (Insulin Chicago). *Diabetes* 32:872–75, 1983
2. Haneda M, Chan SJ, Kwok SCM, Rubenstein AH, Steiner DF: Studies on mutant human insulin genes: identification and sequence analysis of a gene coding (Ser B24) insulin. *Proc Natl Acad Sci USA* 80:6366–70, 1983
3. Shoelson S, Haneda M, Blix P, Nanjo A, Sanke T, Inouye K, Steiner DF, Rubenstein AH, Tager HS: Three mutant insulins in man. *Nature* (Lond) 302:540–43, 1983
4. Sakura H, Ywamoto Y, Sakamoto Y, Kuzuya T, Hirata H: Structurally abnormal insulin in a diabetic patient: characterization of a mutant insulin A3 (Val-Leu) isolated from the pancreas. *J Clin Invest* 78:1666–72, 1986
5. Shibasaki Y, Kawakami T, Kanazawa Y, Akanuma Y, Takakuku F: Post-translational cleavage of proinsulin is blocked by a point mutation in familial hyperproinsulinemia. *J Clin Invest* 76:378–80, 1985
6. Chan SJ, Seino S, Gruppuso PA, Schwartz R, Steiner DF: A mutation in the A chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. *Proc Natl Acad Sci USA* 84:2194–97, 1987
7. Ohashi H, Kannemitsu M, Omori M: A case of familial hyperproinsulinemia with insulin allergy. *Horumon To Rinsho* 34:357–60, 1986 (In Japanese)
8. Hashimoto T, Aso N, Umeda F, Yamashita T, Ishi H, Noda K, Motomatsu T, Nawata H: A new case of familial hyperproinsulinemia. *Endocrinol Jpn* 36:545–52, 1989
9. Barbetti F, Raben N, Kadowaki T, Cama A, Accili D, Gabbay KH, Merenich JA, Taylor SI, Roth J: Two unrelated patients with familial hyperproinsulinemia due to a mutation substituting histidine for arginine at position 65 in the proinsulin molecule: identification of the mutation by direct sequencing of genomic DNA amplified by polymerase chain reaction. *J Clin Endocrinol Metab* 71:164–69, 1990
10. Knowler WC, Pettitt DJ, Bennett PH, Williams RC: Diabetes mellitus in the Pima Indians: genetic and evolutionary considerations. *Am J Epidemiol* 113:144–56, 1981
11. Zimmet P, King H, Taylor R, Raper LR, Balkau B, Borger J, Heriot W, Thoma K: The high prevalence of diabetes mellitus, impaired glucose tolerance and diabetic retinopathy in Nauru—the 1982 survey. *Diabetes Res* 1:13–18, 1984
12. Gorden P, Hendricks CM, Roth J: Circulating proinsulin-like component in man: increased proportion in hypoinsulinemic states. *Diabetologia* 10:459–74, 1974
13. Ward WK, LaCava EC, Paquette TL, Beard JC, Wallum BJ, Porte D Jr: Disproportionate elevation of immunoreative proinsulin in type II (non-insulin-dependent) diabetes mellitus and in experimental insulin resistance *Diabetologia* 30:698–702, 1987
14. Yoshioka N, Kuzuya T, Matsuda A, Taniguchi M, Iwamoto Y: Serum proinsulin levels at fasting and after oral glucose load in patients with type II (non-insulin-dependent) diabetes mellitus. *Diabetologia* 31:35–60, 1988
15. Temple RC, Carrington CA, Luzio SD, Owens DR, Schneider AE, Sobey WJ, Hales CN: Insulin deficiency in non-insulin-dependent diabetes. *Lancet* 1:293–95, 1989
16. Saad MF, Kahn SE, Nelson RG, Pettitt DJ, Knowler WC, Schwartz MW, Kowalyk S, Bennett PH, Porte D Jr: Disproportionately elevated proinsulin in Pima Indians with non-insulin dependent diabetes mellitus. *J Clin Endocrinol Metab* 70:1247–53, 1990
17. World Health Organization: *Diabetes Mellitus: Report of a WHO Study Group*. Geneva, World Health Org., 1985, p. 9–17 (Tech. Rep. Ser., no. 727)
18. Robbins DC, Shoelson SE, Rubenstein AH, Tager HS: Familial hyperproinsulinemia. *J Clin Invest* 73:714–19, 1984
19. Higuchi R: Simple and rapid preparation of samples for PCR. In *PCR Technology. Principles and Applications for DNA Amplification*. Erlich HA, Ed. New York, Stockton, 1989, p. 31–38
20. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–91, 1987
21. Tager HS, Patzelt C, Assoian RK, Chan SJ, Duguid JR, Steiner DF: Biosynthesis of islet cell hormones. *Ann NY Acad Sci* 343:133–47, 1980
22. Steiner DF, Quinn PS, Chan SJ, Marsh J, Tager HS: Processing mechanisms in the biosynthesis of proteins. *Ann NY Acad Sci* 343:1–16, 1980
23. Gabbay KH, Deluca K, Fisher JN, Mako ME, Rubenstein AH: Familial hyperproinsulinemia: an autosomal dominant defect. *N Engl J Med* 294:911–15, 1976
24. Kanazawa Y, Hayashi M, Ikeuchi M: Familial proinsulinemia: a rare disorder of insulin biosynthesis. *Excerpta Med Int Congr Ser* 468:262–69, 1979
25. Gruppuso PA, Gorden PG, Kahn R, Cornblath M, Zeller P, Schwartz R: Familial hyperproinsulinemia due to a proposed defect in conversion of proinsulin to insulin. *N Engl J Med* 311:629–34, 1984
26. Ullrich A, Dull TJ, Gray A, Brosius J, Sures I: Genetic variation in the human insulin gene. *Science* 209:612–15, 1980
27. Bell GI, Pictet RL, Rutter WJ, Cordell B, Tisher E, Goodman HM: Sequence of the human insulin gene. *Nature* (Lond) 284:26–32, 1980
28. Owerbach D, Nerup J: Restriction fragment length polymorphism of the insulin gene in diabetes mellitus. *Diabetes* 31:275–77, 1982
29. Rotwein PS, Chirgwin J, Province M, Knowler WC, Pettitt DJ, Cordell B, Goodman HM, Permutt MA: Polymorphism in the 5′ flanking region of the human insulin gene: a genetic marker for non-insulin-dependent diabetes. *N Engl J Med* 308:65–71, 1983
30. Knowler WC, Pettitt DJ, Vasquez B, Rotwein PS, Andreone TL, Permutt MA: Polymorphism in the 5′ flanking region of the human insulin gene. *J Clin Invest* 74:2129–35, 1984
31. Bell GI, Horita S, Karam JH: A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 33:176–83, 1984
32. Elbein S, Rotwein P, Permutt MA, Bell GI, Sanz N, Karam JH: Lack of association of the polymorphic locus in the 5′-flanking region of the human insulin gene and diabetes in American Blacks. *Diabetes* 34:433–39, 1988
33. Elbein SC, Corsetti L, Goldgar D, Skolnick M, Permutt MA: Insulin gene in familial NIDDM: lack of linkage in Utah Mormon pedigrees. *Diabetes* 37:569–76, 1988
34. Cox NJ, Epstein PA, Spielman RS: Linkage studies on NIDDM and the insulin and insulin-receptor genes. *Diabetes* 38:653–58, 1989
35. Serjeantson SW, Owerbach D, Zimmet P, Nerup J, Thoma K: Genetics of diabetes in Nauru: effects of foreign admixture, HLA antigens and the insulin-gene-linked polymorphism. *Diabetologia* 25:13–17, 1983
36. Sanz N, Karam JH, Horita S, Bell GI: Prevalence of insulin-gene mutations in non-insulin-dependent diabetes mellitus. *N Engl J Med* 314:1322–23, 1986
37. Sanz N, Horita S, Karam JH, Sanke T, Xiang K, Bell GI: Insulin gene polymorphisms and diabetes mellitus. *J Jpn Diabetic Soc* 30:1140–43