Diabetologia (1992) 35: 261–266

Diabetologia

Detection of mutations in the insulin receptor gene in patients with insulin resistance by analysis of single-stranded conformational polymorphisms

H. Kim[1], H. Kadowaki[2], H. Sakura[1], M. Odawara[1], K. Momomura[1], Y. Takahashi[1], Y. Miyazaki[3], T. Ohtani[4], Y. Akanuma[2], Y. Yazaki[1], M. Kasuga[5], S. I. Taylor[6] and T. Kadowaki[1, 2]

[1] The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo,
[2] Institute for Diabetes Care and Research, Asahi Life Foundation,
[3] Misato Kenwa Hospital, Misato,
[4] Department of Pediatrics, Tokyo Women's Medical College, Tokyo,
[5] The Second Department of Internal Medicine, Faculty of Medicine, Kobe University, Kobe, Japan, and
[6] Diabetes Branch, National Institutes of Health, Bethesda, Maryland, USA

Summary. We analyzed single-stranded conformational polymorphisms to screen for mutations and polymorphisms in the insulin receptor gene in subjects with or without insulin resistance. Using this new technique, we demonstrated the existence of mutations in the insulin receptor gene which we had identified previously. In addition, a new mutation was found in exon 20 of the insulin receptor gene in a patient with moderate insulin resistance associated with morbid obesity, acanthosis nigricans, and polycystic ovary syndrome. The patient was heterozygous for a mutation substituting Leu (C*T*G) for Pro (C*C*G) at codon 1178. Pro^{1178} is a part of a characteristic sequence motif (D^{1150} F^{1151} G^{1152}---A^{1177} P^{1178} E^{1179}) common to many protein kinases. Analysis of single-stranded conformational polymorphisms was also used to estimate the frequency of a polymorphism at codon 1058. The two codons CAC (1058 His) and CAT (1058 His) both had a prevalence of 50 % in 30 Japanese subjects. These data demonstrate that analysis of single-stranded conformational polymorphisms is a simple and sensitive screening method for mutations and polymorphisms in the insulin receptor gene in subjects with or without insulin resistance. Identification of a mutation in the insulin receptor gene in a patient with a moderate degree of insulin resistance associated with morbid obesity suggests that insulin receptor mutations may exist in patients with Type 2 (non-insulin-dependent) diabetes mellitus associated with a moderate degree of insulin resistance.

Key words: Hyperinsulinaemia, tyrosine kinase activity, Type 2 (non-insulin-dependent) diabetes mellitus, obesity, screening.

Mutations in the insulin receptor gene can cause insulin resistance and diabetes mellitus [1–17]. We have previously identified mutations in the insulin receptor gene in patients with genetic forms of extreme insulin resistance such as leprechaunism, the Rabson-Mendenhall syndrome, and type A extreme insulin resistance [1, 2, 6, 10, 11, 15–17]. These mutations were originally identified by cDNA cloning [2, 3, 6, 8, 9] and more recently by methods utilizing the polymerase chain reaction (PCR) and direct sequencing [10–12, 14, 15–17]. However, because these methods require determination of the entire protein coding sequence gene to identify base substitutions, there has been a need for more rapid and efficient techniques.

Recently, Orita et al. reported a new technique for analyzing single-stranded conformational polymorphisms (SSCP) in DNA amplified by PCR [18, 19]. This method is based on the principle that the conformation of a single-stranded DNA molecule depends upon its nucleotide sequence. Even a point mutation can alter the conformation, causing a detectable shift in electrophoretic mobility. In the present study, we have demonstrated that PCR-SSCP analysis can detect variants in the nucleotide sequence of the insulin receptor gene. We successfully detected five mutations and polymorphisms previously identified in the insulin receptor gene. Upon screening five patients with mild and moderate degrees of insulin resistance using PCR-SSCP, we detected a new mutation of the insulin receptor gene in one patient with moderate insulin resistance and obesity.

Subjects, materials and methods

Genomic DNA

Genomic DNA derived from four patients [2, 6, 8, 10, 11] with identified mutations in the insulin receptor gene was isolated from lymphoblast cell lines transformed by the Epstein-Barr virus using standard procedures as previously described [10]. Genomic DNA was also prepared from lymphocytes from venous blood of 35 additional subjects: seven normal control subjects, 23 patients with Type 2 (non-insulin-dependent) diabetes without known hyperinsulinaemia, and five patients with mild to moderate degrees of hyperinsulinaemia. The diagnosis of diabetes was based on the criteria set by the Japan Diabetic Society in 1982 [20]. Four of the five patients with mild to moderate degrees of hyperinsulinaemia had poly-

Table 1. Pairs of primers for amplifying exons of the human insulin receptor gene

Exon	Primer Up Stream (x-2) Down Stream (x-1)	Size of PCR Products (bp)
6 (6-2)	5'AGGCACGTAGCACTGAACA	433
(6-1)	5'-TGTAATGCACTTGAATCATGCTG	
14 (14-2)	5'-TGGACACACTCCCAGATGTGCA	276
(14-1)	5'-ACCATGCTCAGTGCTAAGCA	
16 (16-2)	5'-TCTGCTGGTAAGGGCTGCCA	248
(16-1)	5'-CTCACTCAATGGTGAAGGCA	
17 (17-2)	5'-CCAAGGATGCTGTGTGTAGATAAG	
(17-1A)	5'-TCAGGAAAGCCAGCCCATGTC	317
(17-1B)	5'-TGATGGCAGGTGAAGCCCTTC	273
18 (18-2)	5'-CTGGTGAGTCGAATCACGGA	214
(18-1)	5'-AGCGGGTGCTTCCACCGAGTA	
19 (19-2)	5'-GATCCCAGTGCTGCTGAAACAC	285
(19-1)	5'-ACGGCTCATTATAGACAACTTC	
20 (20-2)	5'-AGGTTAAGAGCGTGTGAACCT	208
(20-1)	5'-GAATTCAAGCCCAGCGTCCAT	
21 (21-2)	5'-TGTTACTACTATCAACTGTC	291
(21-1)	5'-ACCTGTAACATACAGCATGC	

PCR, polymerase chain reaction; bp, base pairs

cystic ovary disease syndrome with fasting immunoreactive insulin levels ranging from 25 μU/ml to 97 μU/ml. One of the four patients is morbidly obese (BMI 34.5 kg/m^2) and one of them is obese (BMI 28.5 kg/m^2) with acanthosis nigricans.

Primers for PCR-mediated amplification of the insulin receptor gene

Primers were chosen in the introns adjacent to each exon [21, 22] (Table 1). Two different sets of primers were used in the present study to amplify exon 17; the second set was chosen within the sequence of exon 17 to exclude the site of polymorphism commonly observed in the normal subjects [6, 11]. For exons 6, 14, 16, 18, 19, 20, and 21, single sets of primers were used.

The oligonucleotides used as primers were synthesized by the phosphoroamidite method with trityl in the DNA synthesizer and purified with oligonucleotide purification columns (Applied Biosystems Inc., Foster City, Calif., USA).

PCR-SSCP analysis of the insulin receptor gene on genomic DNA

Exons 6, 14, 16, 17, 18, 19, 20 and 21 of the insulin receptor genes were amplified by PCR catalyzed by Taq DNA polymerase using genomic DNA as template and ^{32}P-end-labelled oligonucleotides as primers [10, 11, 23].

Following initial denaturation of DNA strands at 94 °C for 5 min, the reactions consisted of 25 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and extension at 70 °C for 90 s [10, 11, 21–23]. The PCR products were diluted 1:10 with loading dye (98% formamide, 20 mmol/l EDTA, 0.05% bromo-phenol-blue, 0.05% xylenecyanol), heated at 80 °C for 5 min, and 1 μl of each sample was loaded onto a 5% polyacrylamide gel (20 × 40 × 0.03 cm, acrylamide: N,N′ bis acrylamide = 49:1). Each sample was applied to four gels, two containing 10% glycerol and two without; one of each type of gel was subjected to electrophoresis at 4 °C and one at room temperature at a constant power of 40 W, completed in 2.5 to 4 h using laminar flow fans. Electrophoresis was performed in a Tris-borate buffer containing 45 mmol/l Tris-borate and 1 mmol/l EDTA. The gels were allowed to dry on filter paper (Whatman 3 mm, Whatman International Ltd, Maidstone, UK) and then autoradiographed with X-ray film using intensifying screens at −70 °C for 12–24 h [18, 19].

Direct sequencing of amplified genomic DNA

The nucleotide sequence of amplified DNA was determined directly by the method of Kadowaki et al. [10, 11].

Results

Detection of a polymorphism in exon 17 in 30 subjects

When ^{32}P-labelled PCR products of exon 17 of the insulin receptor gene were separated by electrophoresis after heat denaturation, they migrated as two ^{32}P-labelled bands as exemplified in lane 1 from a control subject (Fig. 1 a, b). The upper and lower bands represent two complementary single-strand DNAs. As shown in lane 2 in Figure 1 a, both upper and lower ^{32}P-labelled bands from another control subject migrate differently as compared to lane 1. Direct sequencing analysis of the two PCR products revealed a single nucleotide difference CAT (lane 1 and 3, positions A and C) vs CAC (lane 2 and 4, positions B and D) at codon 1058 both encoding histidine [6, 11]. As shown in lane 5–7 (Fig. 1 a), ^{32}P-labelled

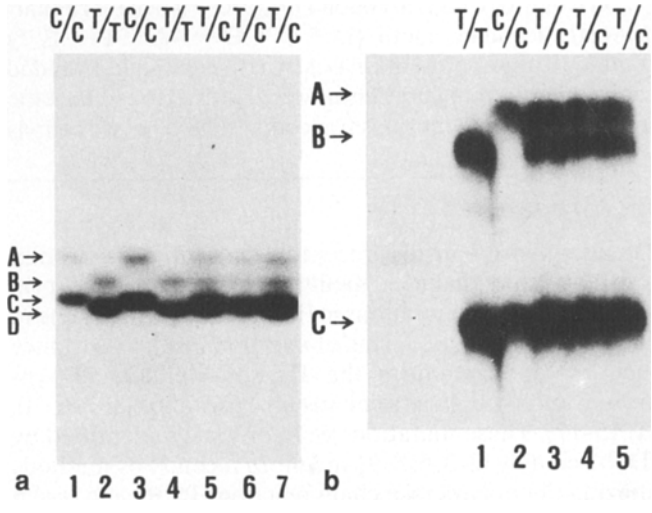

Fig. 1 a, b. A polymorphism in exon 17 detected by neutral polyacrylamide gel electrophoresis without (**a**) and with (**b**) 10% glycerol at room temperature. **a** Two patterns of the polymerase chain reaction (PCR) products in single-stranded conformational polymorphisms analysis from two normal subjects are shown in lanes 1 and 2. Analysis by direct sequencing of these two PCR products revealed a single nucleotide difference CAC (lane 1, A) vs CAT (lane 2, B) at codon 1058 both encoding histidine (6, 11). The subjects in lane 1 and 3 are homozygous for CAC and the subjects in lane 2 and 4 are homozygous for CAT. Subjects shown in lane 5–7 were heterozygous for CAC and CAT. **b** The subject in lane 1 is homozygous for CAT and the subject in lane 2 is homozygous for CAC. The order of the samples in lane 1 and lane 2 is reversed as compared to that in Figure 1 a. Samples shown in lane 3–5 are heterozygous for CAC and CAT

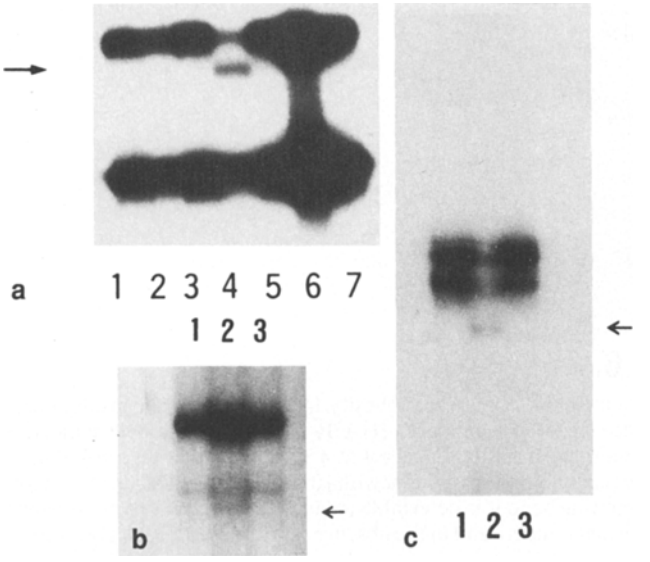

Fig. 2. **a** Detection of known mutations using the second set of primers in exon 17 (17-1B, 17-2) by polymerase chain reaction-single-stranded conformational polymorphisms (PCR-SSCP) analysis. The site of polymorphism at codon 1058 was excluded from the PCR product. In lanes 1, 2, 3, 5, 6 and 7, normal control subjects showed only two bands, whereas in lane 4, which is the sample from a patient with type A syndrome (6), two distinct upper bands were detected. This result is consistent with the fact that this patient has a heterozygous mutation substituting Val for Gly at codon 1008 in exon 17(6) **b** Detection of a known mutation in exon 6 by PCR-SSCP. Under the conditions with 10% glycerol at room temperature, in lane 1 and 3, normal control subjects showed only two bands. In lane 2, two lower bands were observed in the sample from patient leprechaunism/Ark-1 who is heterozygous for a mutation substituting Glu for Lys at codon 460 in exon 6(2) **c** Detection of a known mutation in exon 14 by PCR-SSCP. In lanes 1 and 3, normal control subjects showed only two bands. In lane 2, one of the two upper bands ran slower than those in lane 1 and 3 under the conditions without glycerol at room temperature. The subject in lane 2, was from patient leprechaunism/Minn-1 who was heterozygous for a mutation substituting a stop codon for Arg at codon 897 in exon 14(10)

PCR products from additional control subjects migrated at positions A, B, C, and D suggesting that these subjects' were heterozygous for both CAT and CAC at this position when detected by polyacrylamide gel electrophoresis without glycerol at room temperature. This was confirmed by PCR-direct sequencing (data not shown). PCR-SSCP analysis of the same PCR products were carried out under different conditions (room temperature, 10% glycerol (+)) (Fig. 1 b). Upper bands again migrated at two distinct positions (A and B), while lower bands migrated at only one position (C). This suggests that one nucleotide change at this position affects the electrophoretic mobility of only one of these two complementary single-strand DNAs under these conditions.

Based upon the results of PCR-SSCP, we have calculated the frequency of both alleles. The allele frequency of CAC and CAT at position 1058 was estimated at 50%:50%, respectively, in 30 Japanese subjects. However, there was no difference in the allele frequency between the normal subjects and the subjects with Type 2 diabetes (data not shown).

Detection of known mutations in the insulin receptor gene

The presence of three patterns due to the polymorphism in exon 17 interferred with unequivocal detection of mutations in other regions in this amplified fragment (data not shown). When primers within the sequence of exon 17 were chosen to exclude the site of the polymorphism, we were able to detect the mutations in exon 17 which had been identified in the patients with extreme insulin resistance. Using primers 17-1B and 17-2 (Table 1), we applied the SSCP method to analyse genomic DNA from a patient with type A extreme insulin resistance [6] and a patient with the Rabson-Mendenhall syndrome [11]. When this set of primers was used, the site of polymorphism at codon 1058 was excluded, and only two bands were observed in the sample from normal control subjects (Fig. 2 a, lanes 1–3, 5, 6). However, in one of these samples, two distinct upper bands were detected (Fig. 2 a, lane 4). This is consistent with the fact that the patient was previously shown to be heterozygous for a mutation substituting Val for Gly at codon 1008 in exon 17 [6]. A heterozygous mutation in exon 17 substituting a stop codon for Arg at codon 1000 that we have previously identified [11] was also detected by SSCP under identical conditions (data not shown). Thus, both of the two mutations of exon 17 previously identified were clearly detected under the conditions 4°C without glycerol at. We extended PCR-SSCP analysis to other exons of the insulin receptor gene. In exon 6, under the conditions with 10% glycerol at room temperature, two lower bands were observed in the sample taken from patient leprechaunism/Ark-1 who is heterozygous for a missense mutation substituting Glu for Lys at codon 460 in exon 6 (Fig. 2 b) [2]. In exon 14, under the conditions without glycerol at room temperature, compared with control DNA, one of the two upper bands ran slower in the sample isolated from a patient who was heterozygous for a mutation substituting a stop codon for Arg at codon 897 in exon 14 (Fig. 2 c) [10]. Thus, the SSCP method successfully detected four previously described mutations in exons 6, 14, and 17 of the insulin receptor gene of patients with type A extreme insulin resistance, the Rabson-Mendenhall syndrome, and leprechaunism.

Screening subjects with Type 2 diabetes and insulin resistance for variant sequence in the tyrosine kinase domain of the insulin receptor gene

Genomic DNA was screened for mutations in the tyrosine kinase domain of the insulin receptor gene (exons 16–21) of five patients with mild to moderate insulin resistance and 23 patients with Type 2 diabetes without known hyperinsulinaemia. Electrophoresis was carried out under all four experimental conditions. In analysing amplified DNA from exon 20 of a patient with amenorrhoea, obesity, acanthosis nigricans, hypertrichosis, and moderate degree of insulin resistance, we observed that one of the two lower bands migrated more slowly under the conditions without 10% glycerol at 4°C, in the analysis of exon 20 of the insulin receptor gene (Fig. 3 a, lane 6). Under the conditions with 10% glycerol at 4°C, an aber-

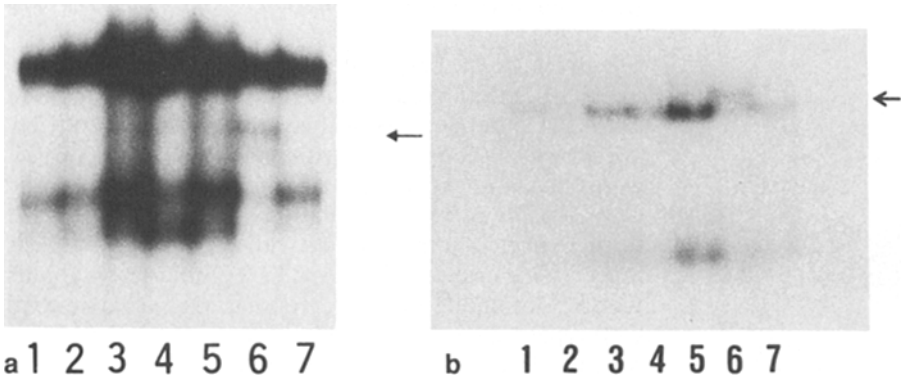

Fig. 3 a, b. Genomic DNA screening for mutations in the insulin receptor gene in patients with mild to moderate insulin resistance. Analysis of exon 20 in the insulin receptor gene by polymerase chain reaction-single-stranded conformational polymorphisms (PCR-SSCP) analysis was performed in patients with mild to moderate insulin resistance (lanes 1–7). The genomic DNA sample in lane 6 was taken from a women with obesity, hyperandrogenism, insulin resistance, acanthosis nigricans (HAIR-AN) and the sample subject to conditions **a** without glycerol at 4°C **b** with 10% glycerol at 4°C. Under conditions without or with 10% glycerol at 4°C, an aberrantly migrating band was detectable in lane 4, whereas it was not obvious at room temperature in the absence or presence of 10% glycerol

rantly migrating band was also detected in this sample (Fig. 3 b, lane 6). Under the conditions at room temperature in the absence or presence of 10% glycerol, we could still discern aberrantly migrating bands in samples from this patient, although alterations in electrophoretic mobility were less evident than at 4°C (data not shown).

We observed minor faint bands with all of the samples only under the conditions without glycerol at 4°C. The relative intensities and the mobilities of these bands depended on the conditions of electrophoresis. We thought that these bands were different conformations of the same sequence sometimes occurring under certain conditions [19].

Independently, by directly determining the sequence of amplified DNA, we demonstrated that this patient is heterozygous for a mutation substituting Leu (C*T*G) for Pro (C*C*G) at codon 1178 in exon 20 (Fig. 4).

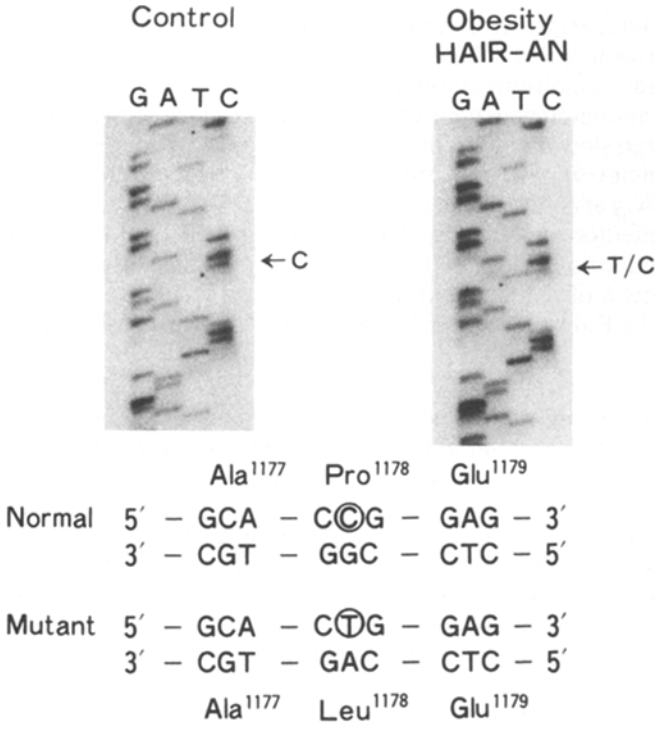

Fig. 4. Polymerase chain reaction direct sequencing of exon 20 in the insulin receptor gene of the patient with an abnormal single-stranded conformational polymorphisms electrophoretic pattern in Figure 3. The nucleotide sequence of amplified exon 20 of the insulin receptor gene was determined directly [10, 11]. The patient is heterozygous for a mutation substituting Leu (C*T*G) for Pro (C*C*G) at codon 1178 in exon 20

Discussion

Predisposition to Type 2 diabetes may partly be due to mutations in the insulin receptor [1]. At least 25 point mutations in the insulin receptor gene have been identified in patients with genetic forms of extreme insulin resistance [2–17]. Many of these mutations have been identified by direct sequencing of PCR products after amplifying each of the 22 exons of the insulin receptor gene using patients' genomic DNA as templates [10–12, 14–17]. However, this technique is quite labor-intensive and time-consuming. Therefore, large-scale screening for mutations in the insulin receptor gene has not been possible.

In recent years, several new techniques for detecting mutations have been developed. These include chemical mismatch cleavage [24] and ribonuclease cleavage [25, 26] at mismatches and denaturing gradient gel electrophoresis (DGGE). However, the sensitivity of cleavage methods has often been reported as unsatisfactory [24–26]. To optimize the sensitivity of the DGGE technique, it is necessary to attach a 40 base pair GC-rich fragment (GC clamp) at the end of one of the primers [27, 28].

In 1989, Orita et al. reported a new simple technique referred to as PCR-SSCP based upon the principle that the conformation and electrophoretic mobility in the non-denaturing gel of single-strand DNA depends exquisitely upon its nucleotide sequence [18, 19]. PCR-SSCP has recently been used to screen for base substitutions in other genetic diseases such as neurofibromatosis type 1 [29] or cystic fibrosis [30]. In the present study, we were able to detect all four mutations and the polymorphisms tested

Table 2. Detectability of mutations and polymorphisms in the human insulin receptor gene by single-stranded conformational polymorphisms under four conditions

Exon	Patient	Codon			Primers	Length (bp)	RT G (−)	RT 10% G (+)	4°C G (−)	4°C 10% G (+)
		#	wild type	variant						
17	Normal	1058	CA*C* (CA*T*)	CA*T* (CA*C*)	17-1A 17-2	317	+	+	−	−
17	Rabson-Mendenhall	1000	*C*GA	*T*GA	17-1B 17-2	273	−	−	+	+
17	Type A	1008	GG*C*	GT*C*	17-1B 17-2	273	−	+	+	+
6	leprechaunism	460	*A*AG	*G*AG	6-1 6-2	433	−	+	+	+
14	leprechaunism	897	*C*GA	*T*GA	14-1 14-2	276	+	−	−	−
20	Obesity HAIR-AN	1178	CC*G*	CT*G*	20-1 20-2	208	−	−	+	+

RT, room temperature; bp, base pairs; HAIR-AN, hyperandrogenism, insulin resistance, acanthosis nigricans; G(−), without glycerol; 10% G(+), containing 10% glycerol; + +, remarkably detectable; +, detectable; −, not detectable

that previously had been identified by either cDNA cloning or direct sequencing of the insulin receptor gene. Using the PCR-SSCP method, the entire procedure including exposure time can be completed within 24 h, and no special equipment is required. Thus, the PCR-SSCP method is a simple, fast, and sensitive method for detecting mutations in the human insulin receptor gene.

We performed electrophoresis of SSCP under four conditions, i.e. at room temperature or 4 °C in the presence or absence of 10% glycerol. The results of the present study suggest that these conditions may affect the sensitivity of the technique differently depending upon the various base substitutions. Thus, although some mutations were preferentially detected under one or two of these conditions, other mutations were preferentially detected under other conditions (Table 2). Therefore, we suggest that it is important to perform PCR-SSCP under all four conditions to maximize the sensitivity in detecting mutations. In addition, as shown in the analysis of mutations in exon 17, choosing oligonucleotide primers to exclude the segment of genomic DNA containing frequent polymorphisms increases the ability of PCR-SSCP to detect mutations in this region.

While screening patients with mild to moderate degrees of insulin resistance using PCR-SSCP, a new mutation was detected in a patient who is a 14-year-old female with obesity, acanthosis nigricans and hyperandrogenism causing polycystic ovary disease syndrome, hypertrichosis, and amenorrhoea. Because of her obesity, this patient differs from lean patients with classical type A insulin resistance [31]. The pattern of her oral glucose tolerance test was within normal range. The fasting insulin level was 93 μU/ml and the fasting blood glucose level was 4.3 mmol/l. Previously, it was suspected that insulin resistance and other clinical features in obese patients with the syndrome of the hyperandrogenism, insulin resistance, and acanthosis nigricans (HAIR-AN) are due to obesity rather than mutations in the insulin receptor gene. Furthermore, this patient's insulin resistance is less than that of some patients with the classified type A syndrome [8]. For example, there was a clear hypoglycaemic response, although subnormal, when the patient was given 0.1 U/kg insulin intravenously.

Substitution of Leu (C*T*G) for Pro (CC*G*) may be a functionally relevant mutation. First, substitution of leucine for proline is not a conservative substitution. Second, Pro^{1178} is a part of characteristic sequence motif ($D^{1150}F^{1151}G^{1152}$ --- A^{1177} P^{1178} E^{1179}) common to many protein kinases [32]. Five missense mutations in the cytoplasmic domain of the insulin receptor gene have been described which decreases the receptor's tyrosine kinase activity [2, 4–6, 15]. Two of these mutations (Arg^{993}→Gln[4], Gly^{1008}→Val[6]) are in the region of the ATP binding domain and three of these (Thr^{1134}→Ala[2], Ile^{1153}→Met[15], Trp^{1200}→Ser[5]) cluster near the autophosphorylation sites (Tyr^{1158}, Tyr^{1162}, Tyr^{1163}). The Pro^{1178}→Leu mutation is also located near the autophosphorylation sites. These results are consistent with the notion that these two regions of the cytoplasmic domain of the insulin receptor gene are fundamental for receptor function. The actual functional impact of this mutation is currently being investigated in this laboratory.

Identification of a missense mutation in the insulin receptor gene in this type of patient raises the possibility that mutations in the insulin receptor gene may be more common than was originally supposed in patients with a mild to moderate degrees of insulin resistance such as obesity, acanthosis nigricans, polycystic ovary syndrome and also Type 2 diabetes [33]. Using PCR-SSCP as described in the present study, it will be possible to investigate a much large number of patients than have been studied to date. Eventually, it should become possible to estimate the frequency of mutations in the insulin receptor gene, and to determine whether mutations in the insulin receptor gene contribute to the pathogenesis of common disease such as Type 2 diabetes.

Acknowledgements. We thank Drs. O. Isozaki and T. Tsushima (Tokyo Women's Medical College) for isolating genomic DNA from the patient. We also thank Dr. K. Hayashi (National Cancer Center Research Institute), Dr. O. Koshio (Institute for Diabetes Care and

Research, Asahi Life Foundation), Drs. K. Tobe, H. Tamemoto, Y. Kaburagi, K. Ueki, M. Kishimoto and K. Muraoka (The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo) for help and useful discussions. This work was supported by the Juvenile Diabetes Foundation International research grant 190831 to T. Kadowaki.

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Received: 13 August 1991
and in revised form: 5 November 1991

Dr. T. Kadowaki
The Third Department of Internal Medicine
Faculty of Medicine
University of Tokyo
Hongo 7-3-1 Bunkyo-ku
Tokyo 113
Japan