Amplification and Analysis of Promoter Region of Insulin Receptor Gene in a Patient With Leprechaunism Associated With Severe Insulin Resistance

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A patient with leprechaunism associated with severe insulin resistance was studied to identify the molecular and genetic basis for insulin resistance. Insulin binding and surface labeling of transformed lymphocytes prepared from the patient showed a significantly decreased insulin receptor number on the cell surface. Southern blot analysis of the insulin receptor gene showed no evidence of large insertions or deletions. Furthermore, direct sequencing of all 22 exons and exon-intron junctions of the insulin receptor gene failed to show any missense mutations, nonsense mutations, or mutations at exon-intron junctions. However, Northern blot analysis indicated significantly decreased insulin receptor mRNA expression in the patient's cells. Moreover, restriction endonuclease digestion of the amplified cDNA suggested that the expression levels of one allele were less efficient than the other. These findings suggested that the regulatory region of the insulin receptor gene might have abnormalities. Therefore, we examined the 5′ flanking region of the insulin receptor gene. Southern blot analysis showed no major deletions or insertions between positions −1,823 and −2 relative to the translation initiation site. A 5′ flanking region of the insulin receptor gene spanning positions −881 ~ +7 was amplified by polymerase chain reaction (PCR) and introduced into a reporter plasmid carrying the human growth hormone (hGH) gene. The nucleotide sequence of the amplified fragment showed two polymorphic sites at positions −603 and −500 in the patient, as well as in normal subjects. No other abnormal sequence was found in the patient. Promoter activity measured by hGH expression in transfected mouse L cells was not influenced by the polymorphism at position −603 located in a cluster of GC boxes. These results indicated that the 5′ flanking promoter region of the insulin receptor gene could be analyzed with the use of PCR, and that the decreased expression of insulin receptor gene in a leprechaun patient was not due to alteration of the nucleotide sequence in the examined promoter region, but possibly in another regulatory region of the insulin receptor gene.

STUDIES ON THE INSULIN receptor gene in patients with the genetic syndrome of extreme insulin resistance have recently disclosed a number of alterations in the insulin receptor gene, such as missense mutations, nonsense mutations, deletions, and insertions.[1] All the identified nucleotide changes are expected to cause an altered primary amino acid sequence of insulin receptor molecules. However, in some patients with associated decreased insulin binding, no alterations in the coding region of the insulin receptor gene have been identified, and decreased insulin receptor mRNA expression due to an unknown *cis*-acting abnormality in the regulatory region of the insulin receptor gene has been postulated.[2,3] The 5′ flanking region of the insulin receptor gene plays an important role in the efficient expression of the insulin receptor gene,[4-7] and an abnormality in this region is one of the candidate explanations for decreased expression of the gene.

Previous reports on the cloned 5′ flanking region of the insulin receptor gene showed extremely high GC content and no TATA box or CAAT box in the region. The 877-base pair (bp) region between the *Hin*dIII site and the translation initiation site has been reported to be almost sufficient for maximal promoter activity.[6] In particular, a cluster of four GC boxes around position −600 relative to the translation initiation site appears to be most important for efficient expression of the insulin receptor gene, and it has been reported that Sp1 protein actually binds to this site.[8] However, transcription factors other than Sp1 have also been suggested to affect transcription.[9-13] In previous studies, several differences in the nucleotide sequence of the 5′ flanking region of the insulin receptor gene are reported; it is not confirmed whether some of these differences actually exist as a polymorphism, nor is it investigated whether such differences affect promoter activity.

Recently, polymerase chain reaction (PCR) has been widely used to identify mutations in the insulin receptor gene.[1] However, all previous reports on the 5′ flanking region of the insulin receptor gene have been derived from studies on genomic DNA clones. Amplification of a DNA sequence that has a high GC content like the 5′ flanking region of the insulin receptor gene has usually been difficult with the standard method.[14] In this report, we were able to amplify the 5′ flanking region of the insulin receptor gene with the use of a reaction mixture containing 7-deaza deoxyguanosine triphosphate. We amplified the insulin receptor gene between positions −881 and +7 of a patient with leprechaunism associated with extreme insulin resistance and decreased insulin gene expression. The amplified DNA fragment was introduced into a plasmid carrying the human growth hormone (hGH) gene, and the nucleotide sequence and promoter activity of the amplified DNA were examined.

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SUBJECT AND METHODS

Subject

A 3-month-old female patient with leprechaunism associated with extreme insulin resistance who exhibited extremely decreased insulin receptor binding (<20% of normal) to red blood cells, cultured fibroblasts, and Epstein Barr virus–transformed lymphocytes was reported previously.[15]

Materials

Reporter plasmid p0GH was obtained from Nichols Institute (San Juan Capistrano, CA), modified T7 DNA polymerase (Sequenase Ver 2.0) from USB (Cleveland, OH), Taq DNA polymerase from Perkin-Elmer/Cetus (Emeryville, CA), DEAE-dextran from Pharmacia (Uppsala, Sweden), and chloroquine from Sigma Chemical (St Louis, MO). Anti–insulin-receptor antibody was obtained from a patient with type B insulin resistance syndrome as described previously.[16] Pansorbin was obtained from Calbiochem-Behring (La Jolla, CA). Synthetic oligonucleotides, T4 polynucleotide kinase, and restriction endonucleases were purchased from Takara (Kyoto, Japan). Superscript reverse transcriptase and random hexamers were purchased from GIBCO/BRL (Gaithersburg, MD). [^{125}I]Na was purchased from New England Nuclear (Boston, MA), and [γ-^{32}P]adenosine triphosphate (6,000 Ci/mmol, 1 Ci = 37 GBq) from Amersham (Buckinghamshire, UK). All other chemicals were reagent grade.

Surface Labeling of Transformed Lymphocytes

Epstein Barr virus–transformed lymphocytes (1×10^8 cells) were iodinated as previously described.[17] The cells were solubilized in a solubilizing buffer containing 1% Triton X-100. After ultracentrifugation, soluble material was immunoprecipitated with anti–insulin-receptor antibodies and Pansorbin. Proteins were dissolved in a sample buffer[18] and analyzed by sodium dodecyl sulfate-7.5%–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and autoradiography.[19]

Southern Blot Analysis of Insulin Receptor Gene

Genomic DNA (10 μg) prepared from transformed lymphocytes was completely digested with either *Ban*I, *Ban*II, *Bgl*II, or *Hin*dIII overnight at 37°C and electrophoresed on a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a [^{32}P]deoxycytidine triphosphate–labeled, *Sac*I-excised, 3,787-bp insulin receptor cDNA probe extending from the 330th amino acid to the 3′ untranslated region. The membrane was washed, followed by autoradiography.

Direct Sequencing of Insulin Receptor Gene

Direct sequencing of all 22 exons of the insulin receptor gene was performed essentially as described previously.[17] Results were compared with the nucleotide sequence reported by Seino et al.[20]

Northern Blot Analysis of Insulin Receptor mRNA

Total RNA was isolated from transformed lymphocytes by the acid guanidium-phenol-chloroform method. Polyadenylated RNA was concentrated with an oligo(dT)-affinity column. Poly(A)$^+$ RNA (10 μg) was incubated with 10 mmol/L phosphate buffer (pH 6.5) in the presence of 0.5 mol/L glyoxal at 50°C for 60 minutes, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. The immobilized RNA was hybridized with an insulin receptor cDNA probe and autoradiographed. The probe was removed, and the membranes were rehybridized with the β-actin probe.

Reverse Transcriptase PCR and Restriction Endonuclease Digestion

Total mRNA was extracted from transformed lymphocytes. First-strand cDNA was synthesized with Superscript reverse transcriptase and random hexamers, followed by amplification using a sense primer (5′ACAACCAGAACCTAAGGCA3′) and an antisense primer (5′TTCAGGCATGGTCCTCGCA3′). The amplified 1,215-bp fragment was digested with *Eco*T22I overnight at 37°C,

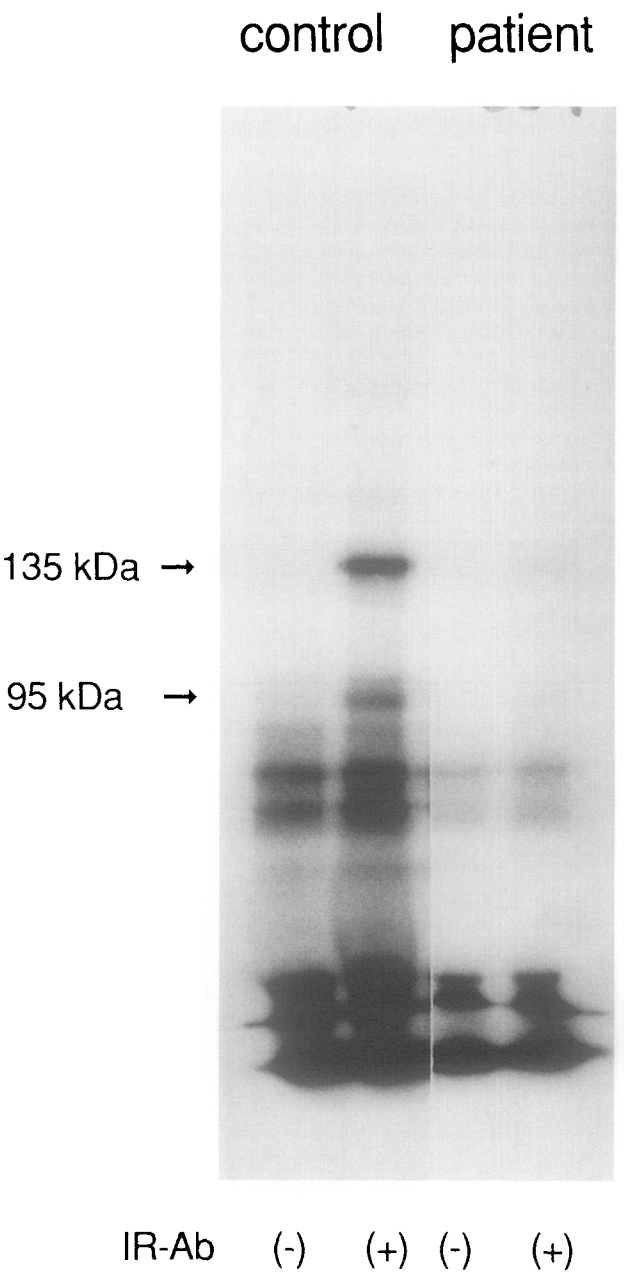

Fig 1. Surface labeling of transformed lymphocytes. Transformed lymphocytes (1×10^8 cells) prepared from either control subjects or the patient were iodinated, solubilized, immunoprecipitated with anti–insulin-receptor antibodies (IR-Ab), and analyzed by SDS-PAGE.

followed by electrophoresis on a 2% agarose gel and ethidium bromide staining.

Southern Blot Analysis of 5′ Flanking Region of Insulin Receptor Gene

Genomic DNA (40 μg) prepared from white blood cells of the patient and a normal subject was completely digested with restriction endonucleases *Bgl*II and *Nco*1, which were expected to recognize positions −1,823 and −2 of the 5′ flanking region of the insulin receptor gene, respectively, according to a sequence reported by Seino et al.[6] It was then electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a mixture of two labeled oligonucleotide probes (5′GGCGCAGAGTCCCTTCCTAGG3′ and 5′CTCGGGGCCCGGAGCTCCGCG3′), which were complementary to positions −546 ~ −526 and −295 ~ −275, respectively.

Amplification of 877-bp Promoter Region of Insulin Receptor Gene

According to the sequence reported by Seino et al,[6] a sense primer (5′CCACCTCGAGTCACCAAAATA3′) and an antisense primer (5′ATAGGATCCGTGCCAATGGCTGCGGGAGCG3′) were synthesized for enzymatic amplification of the 5′ flanking region of the insulin receptor gene. The upstream primer was complementary to position −881 ~ −861 and included a recognition site for *Xho*I at position −877. The downstream primer was complementary to position −13 ~ +7, to which the recognition site for *Bam*HI was added. The initiation codon ATG of the antisense primer was replaced by ATT so as not to start translation at this site.

Genomic DNA was digested by restriction endonuclease *Rsa*I and denatured by NaOH before amplification in a total volume of 100 μL reaction mixture containing 2.5 μg of the template DNA, 50 pmol of each upstream and downstream oligonucleotide primer, 50 mmol/L KCl, 10 mmol/L Trishydrochloride (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.01% (wt/vol) gelatin, 1.25 mmol/L each of deoxyadenosine triphosphate, deoxycytidine, triphosphate, deoxyribosylthymine triphosphate, and 7-deaza-2′-deoxyguanosine, and 5 U Taq DNA polymerase.[14] Amplification was performed for 25 cycles, and each cycle consisted of incubation for 60 seconds at 94°C for denaturation, 60 seconds at 55°C for annealing, and 180 seconds at 72°C for primer extension. Amplified DNA was then amplified again by a standard PCR method for 15 cycles.

Subcloning and Sequencing of Amplified DNA

Following digestion with *Xho*I and *Bam*HI, amplified DNA was introduced into the *Sal*I-*Bam*HI site of the hGH reporter plasmid p0GH.[21] Sequencing of the plasmid was performed by a dideoxy chain-termination method using modified T7 DNA polymerase (Sequenase Ver 2.0). For each subject, more than three clones were sequenced, and additional clones were sequenced when variations of nucleotide sequence were found.

Measurement of Promoter Activity

The constructed plasmid was transfected into mouse L cells by a DEAE-dextran method as previously described.[17] Mouse L cells (2×10^5) were transfected with 5 μg of the constructed plasmid, and the cells were cultured for 120 hours. hGH that accumulated in the culture medium was measured by radioimmunoassay.

RESULTS

Surface Labeling of Transformed Lymphocytes

To determine whether decreased insulin binding to transformed lymphocytes obtained from the leprechaun patient was due to decreased receptor number or decreased binding affinity, 1×10^8 transformed lymphocytes were

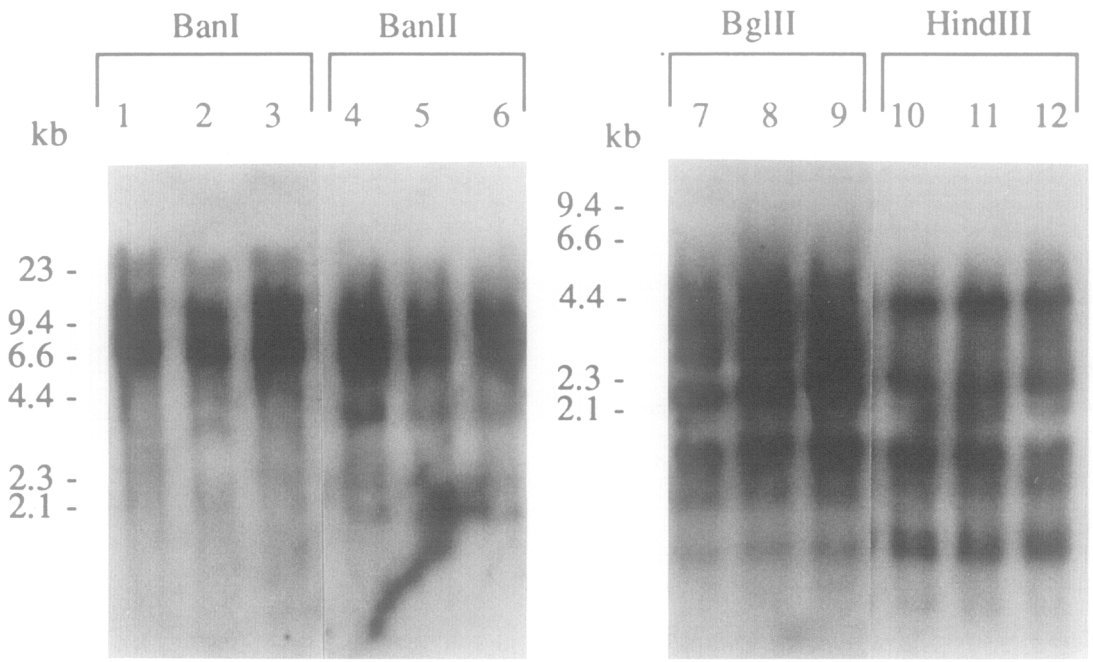

Fig 2. Southern blot analysis of the insulin receptor gene. Genomic DNA prepared from transformed lymphocytes of the patient (lanes 1, 4, 7, and 10) or two normal subjects was completely digested with either *Ban*I, *Ban*II, *Bgl*II, or *Hin*dIII overnight at 37°C, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with labeled insulin receptor cDNA probe.

iodinated, solubilized, immunoprecipitated with anti–insulin-receptor antibodies, and analyzed by SDS-PAGE (Fig 1). The intensity of labeled insulin receptor subunits at 135 kd (α-subunit) and 95 kd (β-subunit) of the patient was significantly decreased compared with that of a normal subject, indicating that decreased insulin binding of the patient's cells was due to decreased insulin receptor number on the cell surface.

Southern Blot Analysis of Insulin Receptor Gene

To eliminate the possibility of large deletions or insertions in the insulin receptor gene, genomic DNA prepared from the patient's transformed lymphocytes was digested with various restriction endonucleases, followed by Southern blot hybridization with insulin receptor cDNA probe. Since the probe excluded the portion of cDNA encoding the *N*-terminal portion of the receptor, a small deletion or rearrangements in that portion may not be excluded. However, digestion patterns of all the restriction endonucleases were the same as those observed for normal subjects (Fig 2).

Exons of Insulin Receptor Gene

All 22 exons of the insulin receptor gene were amplified and directly sequenced. Results showed several silent polymorphisms in the nucleotide sequence (Table 1), but no alterations in the amino acid sequence. Exon-intron junctions were also shown to be normal.

Northern Blot Analysis of Insulin Receptor mRNA

Insulin receptor gene expression in transformed lymphocytes of the patient was estimated with Northern blot analysis (Fig 3). The relative amount of insulin receptor mRNA as compared with control β-actin mRNA was significantly decreased in the patient's cells as compared with normal lymphocytes. Thus, decreased insulin receptor gene expression was suggested to be responsible for the decreased insulin receptor number in the patient's cells.

Relative Amount of mRNA From Each Allele

Since sequencing of genomic DNA showed a heterozygous silent polymorphism at Ala^{523} (GCG and GCA) in exon 8 (Table 1), we used this site to assess the relative amount of mRNA expressed from each allele. A 1,215-bp fragment amplified from first-strand cDNA was digested with restriction endonuclease *Eco*T22I that recognized 5′ATGCAT3′. As shown in Fig 4, the fragment obtained from a normal subject was completely digested to give rise to two fragments (855 bp and 360 bp), indicating that the subject was homozygous for GCA Ala^{523}. In the case of the patient's father, the amplified fragment was not digested at all, consistent with the observation that the father was homozygous for GCG Ala^{523}. As seen in Fig 4, lane 5, the amplified fragment from the patient's mother was partially digested, although more than half of the fragment remained undigested. In contrast, more than half of the amplified fragment from the patient was digested (Fig 4, lane 3). Since direct sequencing of exon 8 showed that the mother and the patient were heterozygous for the polymorphism, the different digestion pattern indicated the differ-

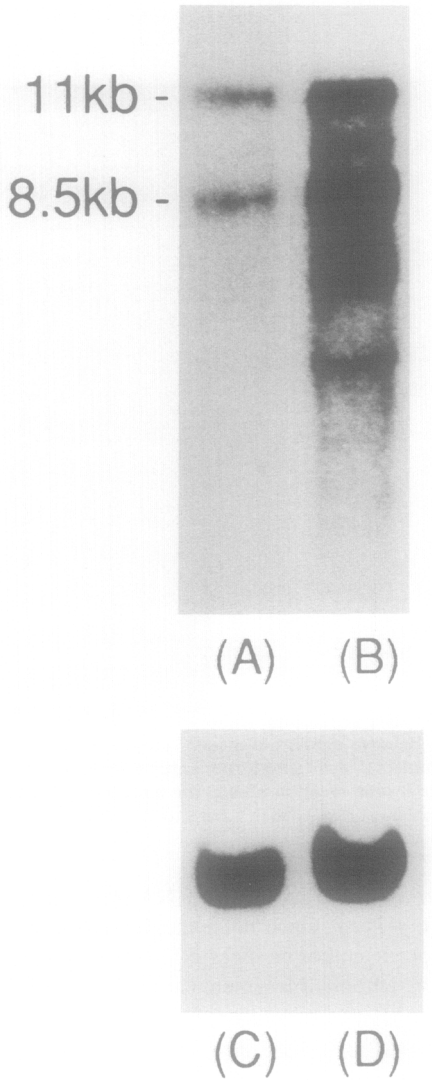

Fig 3. Northern blot analysis of insulin receptor mRNA. Poly(A)⁺ RNA prepared from transformed lymphocytes of either the patient (A and C) or a control subject (B and D) was electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with labeled insulin receptor cDNA probe (A and B). The same membrane was rehybridized with β-actin probe (C and D). Representative data for three independent experiments are shown.

Table 1. Silent Polymorphisms in the Coding Region of the Insulin Receptor Gene Found in a Patient With Leprechaunism

Exon	Amino Acid	Seino et al[17]	Patient
3	Gln^{276}	CAA	CAG/CAG
8	Asp^{519}	GAT	GAC/GAC
8	Ala^{523}	GCG	GCG/GCA
17	Val^{985}	GTC	GTG/GTG

NOTE. Nucleotide sequences are compared with those reported by Seino et al.[20]

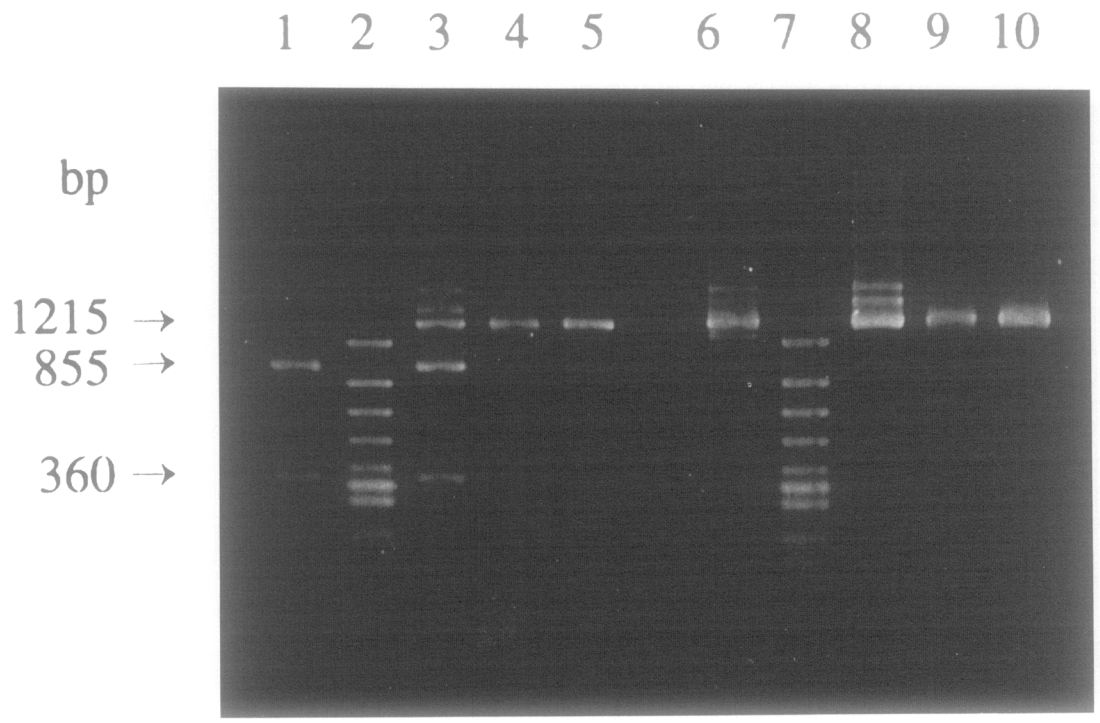

Fig 4. Relative amount of insulin receptor mRNA from each allele. First-strand cDNA prepared from transformed lymphocytes of either a control subject (lanes 1 and 6), the patient (lanes 3 and 8), the father (lanes 4 and 9), or the mother (lanes 5 and 10) was amplified using primers specific to insulin receptor cDNA. The amplified 1,215-bp fragment was digested with restriction endonuclease *Eco*T22I that recognizes silent polymorphism at Ala^{523} (lanes 1 and 3 to 5). Undigested samples (lanes 6 and 8 to 10) and a size marker, χϕ174 *Hin*cII digest (lanes 2 and 7), are also shown.

ent ratio of insulin receptor mRNA transcribed from each allele. In general, since heteroduplex DNA formed in the PCR process cannot be digested by the enzyme, more than half of the amplified fragments are not cleaved if the initial concentrations of mRNA from each allele are equal. Therefore, one of the patient's alleles containing GCG Ala^{523} seems to be expressed less efficiently than the other allele containing GCA Ala^{523}.

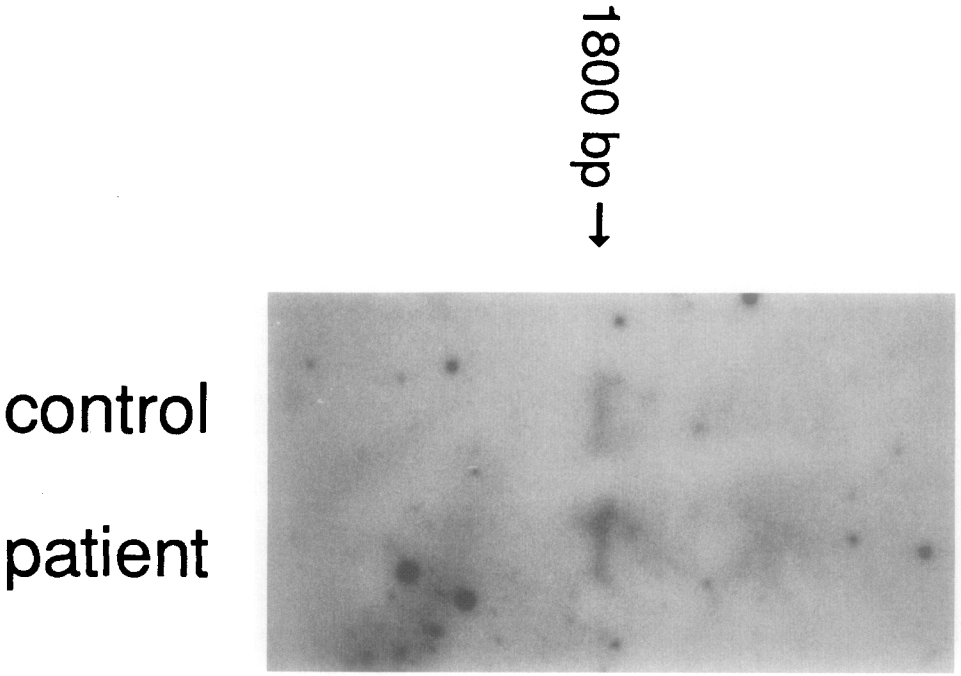

Fig 5. Southern blot analysis of promoter region of the normal subject and the patient. Genomic DNA was digested with restriction endonucleases *Bgl*II and *Nco*I, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with oligonucleotide probes containing promoter sequences.

Southern Blot Analysis of 5′ Flanking Region of Insulin Receptor Gene

To examine whether the approximately 1,800-bp 5′ flanking region of the insulin receptor gene had major abnormalities in the patient, genomic DNA was completely digested with two restriction endonucleases, *Bgl*II and *Nco*I, which were expected to recognize positions −1,823 and −2, respectively, and was hybridized with oligonucleotide probes complementary to sequences in the promoter region. A single band of approximately 1,800 bp was observed for the patient (Fig 5), indicating that this region had no large deletion or insertion.

Nucleotide Sequence and Promoter Activity of 877-bp 5′ Flanking Region of Insulin Receptor Gene

An 877-bp 5′ flanking region of the insulin receptor gene was amplified from genomic DNA and subcloned into p0GH. Clones obtained from the patient had no aberrant nucleotide sequences as compared with those obtained from two normal subjects, and most of the nucleotide sequence was identical to that reported by Seino et al.[6] In the patient and two normal subjects, clones that had either A or G at positions −603 (Fig 6) and −500 were found (Table 2). These variations had been found in previous studies on genomic clones (Table 2). Therefore, these were

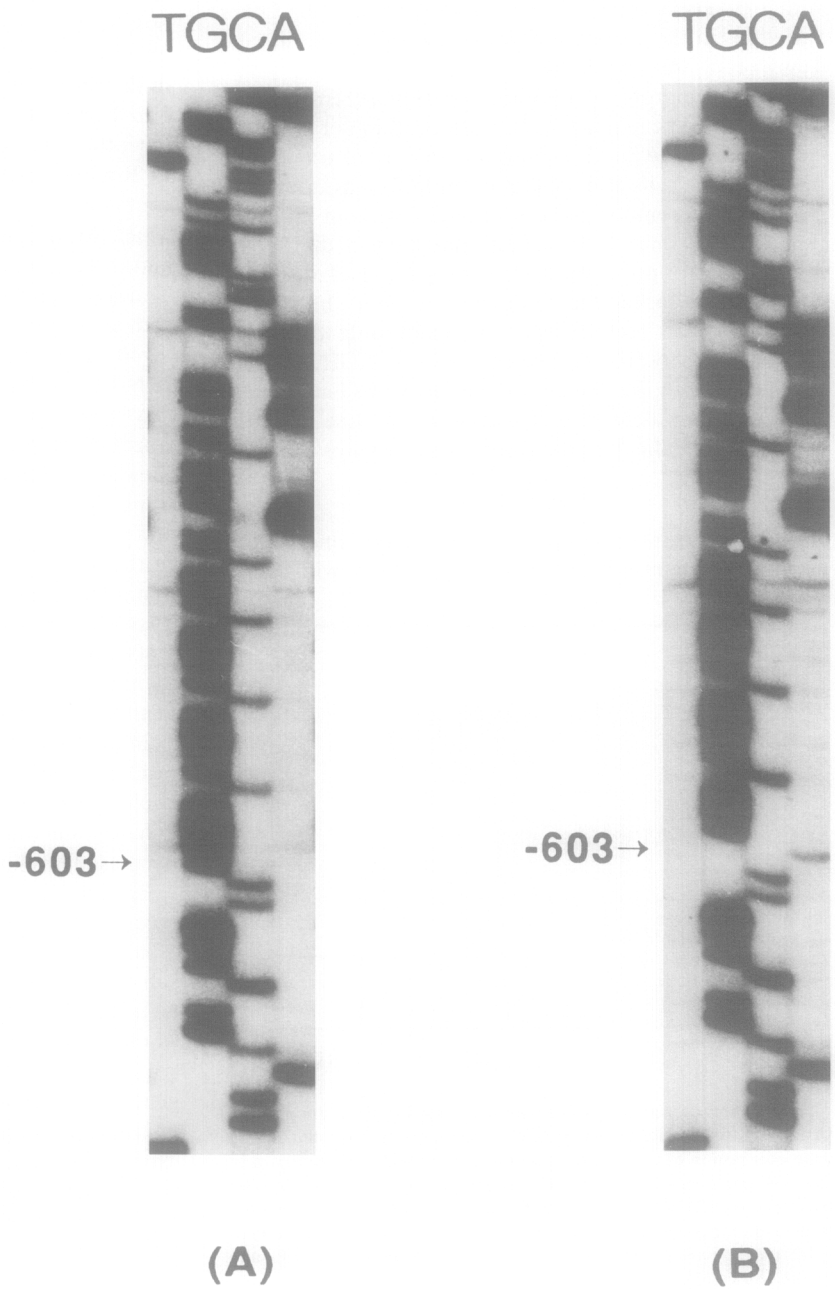

Fig 6. Partial nucleotide sequence of the 5′ flanking region of the insulin receptor gene. The promoter region (877 bp) of the patient was amplified by PCR, introduced into p0GH, and then sequenced. Two clones (A and B) containing G and A, respectively, at position −603 are shown.

Table 2. Two Polymorphisms of the Promoter Region of the Insulin Receptor Gene

	Current Study			Previous Studies	
Position	Patient	Normal No. 1	Normal No. 2	Araki et al[4]	Seino et al[6] and Tewari et al[7]
−603	A/G	A/G	A/G	G	A
−500	A/G	A/G	A/G	A	G

Note. Numbering of position indicates relative bp from translation initiation codon. Nucleotide sequences are compared with those reported by Araki et al,[4] Seino et al,[6] and Tewari et al.[7]

regarded as polymorphisms, and the patient and the normal subjects were heterozygous for these polymorphisms. With regard to position −456, all the clones had C, in agreement with previous reports[4,5,7,22] except that of Seino et al.[6] Other variations in the sequence reported by Mamula et al[5] and Tewari et al[7] were not found.

Since polymorphism at position −603 in a cluster of four GC boxes had been postulated to affect promoter activity,[23] clones carrying A or G at position −603 were transfected into mouse L cells and expression of hGH was examined. Background promoter activity of p0GH without an insertion was less than 10% of that contained in the 877-bp insulin receptor promoter (data not shown), and a deletion construct that lacked the upstream portion to the *Hin*dIII site at position −498 showed a significantly decreased promoter activity (Fig 7). The expression of hGH in L cells transfected with the clones with either A or G at position −603 was equivalent (Fig 7), indicating that the polymorphism may not affect insulin receptor gene expression.

DISCUSSION

In this study, we investigated the molecular and genetic mechanism for insulin resistance in a patient with leprechaunism. Insulin binding and surface labeling of the patient's transformed lymphocytes demonstrated decreased receptor number on the cell surface. No major deletion or insertion in the insulin receptor gene was detected by Southern blot analysis. Furthermore, direct sequencing of all 22 exons of the insulin receptor gene failed to show any missense mutations, nonsense mutations, or mutations at exon-intron junctions. However, Northern blot analysis indicated decreased expression of insulin receptor mRNA in the patient's cells. Moreover, expression of one allele was less efficient than that of the other allele. Therefore, the regulatory region of the insulin receptor gene was postulated to have unidentified abnormalities that led to decreased expression of the insulin receptor gene. Southern blot analysis of the 5′ flanking region of the insulin receptor gene showed no major deletion or insertion in the region of approximately 1,800 bp between *Bgl*II and *Nco* I sites. The 877-bp 5′ flanking region of the insulin receptor gene was successfully amplified with the use of PCR and cloned into a reporter plasmid. In this region are contained two potential Sp1 binding-site clusters, one of which, located near −600, has been reported to bind Sp1 and to be important for efficient expression of the gene.[8] Transcription factors other than Sp1 have been reported to activate insulin receptor gene expression. Sequences near −540 and −510, which are recognized by the factors IRNF-1 and IRNF-2, respectively,[10] are also contained in this region. However, the nucleotide sequence of the cloned fragment of the patient's insulin receptor gene failed to show any difference from that of normal subjects. Recently, transcription factors, CAAT/enhancer binding protein, and nuclear factor-1 have been reported to bind to a region further upstream than position −877 and also to sites in the first intervening sequence of the insulin receptor gene.[9,11,12] Two AT-rich sequences, −1,740 ~ −1,784 and −782 ~ −800, have also been reported to bind nuclear factors identified in differentiated BC3H-1 cells.[13] Therefore, modifications in these sequences other than the sequence examined in this study may be responsible for the decreased insulin receptor gene expression in the patient. Alternatively, gene mutations that make mRNA unstable and enhance degradation of mRNA might account for the decreased insulin receptor mRNA level.

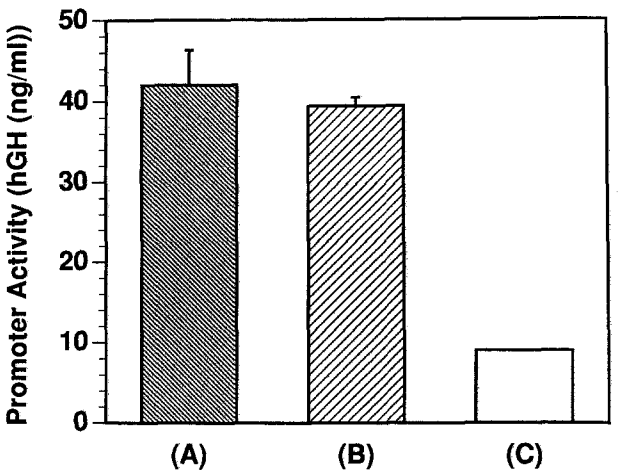

Fig 7. Promoter activity of two clones prepared from the patient. (A and B) Clones indicated in Fig 6. The plasmid containing the 877-bp promoter region of the insulin receptor gene introduced upstream to the hGH gene was transfected into mouse L cells. At 72 hours after transfection, hGH that accumulated in culture medium was measured. Values are the mean ± SEM of four transfections. Promoter activity of a deletion construct that lacks the upstream portion to the *Hin*dIII site is also shown (C).

By sequencing the 877-bp promoter region of the insulin receptor gene, we detected polymorphisms only at positions −603 and −500 in the leprechaun patient and two normal subjects. Therefore, the sequence of this region appears to be highly conserved. Studies on a larger number of subjects would be necessary to confirm if other variations of the sequence in the 5′ flanking region reported previously on genomic clones actually exist as polymorphisms. Polymorphism at position −603 has been postulated to affect gene expression, since this polymorphism is located in the GC box cluster near position −600 and possibly affects Sp1 binding to the GC boxes. However, we found no significant difference in promoter activities between clones that had G and A at this position. However, it would still be possible

that the effect of the polymorphism on insulin receptor gene expression may vary in insulin-sensitive cells in vivo, and the polymorphism may predispose certain populations to develop insulin resistance.

In summary, we were able to examine the 877-bp promoter region of the insulin receptor gene with the use of PCR. Decreased insulin receptor gene expression in a patient with leprechaunism may be due to defect(s) in a regulatory region different from that examined in this study. Further characterization of the regulatory region and the mechanism of decreased expression of the insulin receptor gene in this patient remains to be demonstrated.

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