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Diverse Growth Hormone Receptor Gene Mutations in Laron Syndrome

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Summary

To better understand the molecular genetic basis and genetic epidemiology of Laron syndrome (growth-hormone insensitivity syndrome), we analyzed the growth-hormone receptor (GHR) genes of seven unrelated affected individuals from the United States, South America, Europe, and Africa. We amplified all nine GHR gene exons and splice junctions from these individuals by PCR and screened the products for mutations by using denaturing gradient gel electrophoresis (DGGE). We identified a single GHR gene fragment with abnormal DGGE results for each affected individual, sequenced this fragment, and, in each case, identified a mutation likely to cause Laron syndrome, including two nonsense mutations (R43X and R217X), two splice-junction mutations, (189–1 G to T and 71+1 G to A), and two frameshift mutations (46 del TT and 230 del TA or AT). Only one of these mutations, R43X, has been previously reported. Using haplotype analysis, we determined that this mutation, which involves a CpG dinucleotide hot spot, likely arose as a separate event in this case, relative to the two prior reports of R43X. Aside from R43X, the mutations we identified are unique to patients from particular geographic regions. Ten GHR gene mutations have now been described in this disorder. We conclude that Laron syndrome is caused by diverse GHR gene mutations, including deletions, RNA processing defects, translational stop codons, and missense codons. All the identified mutations involve the extracellular domain of the receptor, and most are unique to particular families or geographic areas.

Introduction

Laron syndrome (LS) is an autosomal, recessively inherited condition with a phenotype similar to that of severe congenital growth hormone (GH) deficiency, except that GH levels are normal or elevated (Laron et al. 1966). Affected individuals have severely short stature, characteristic facies with frontal bossing, truncal obesity, hypoglycemic episodes, and no response to treatment with exogenous GH (Laron et al. 1966). Approximately 140 cases have been reported since 1966, with concentrations of cases among Oriental-Jewish individuals (27 cases) (Laron 1974) and in an isolated population of Spanish descent from the Andes of southern Ecuador (56 cases) (Rosenbloom et al., in press). Sporadic cases have been identified throughout the world (Laron 1974).

The mechanisms by which GH causes growth are incompletely understood. Fetal and early postnatal growth proceeds essentially normally in the absence of pituitary GH (Reid 1960). GH responsiveness develops gradually thereafter (Albertsson-Wikland and Isaksson 1976), with skeletal growth mediated partly by GH-dependent factors produced in the liver, such as insulin-like growth factor-I (Salmon and Daughaday 1957), and partly by GH's direct growth-promoting effects on chondrocytes (Isaksson et al. 1985).

Whereas the unresponsiveness to exogenous GH in LS patients could be due to mutations at various levels

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in the pathway of GH action, the GH receptor (GHR) is the leading candidate for search of mutations. GH initiates cellular responses by interacting with GHRs, which are present at high levels in the liver and also in connective tissue, cartilage, lymphopoietic tissue, and adipose tissue (Isaksson et al. 1985). The GHR is a transmembrane protein consisting of an extracellular domain that includes GH-binding sites, a single transmembrane domain, and a cytoplasmic domain (Leung et al. 1987). On GH binding, dimerization of the extracellular domains from two GHR molecules occurs, yielding a stoichiometry of two GHRs to one GH molecule (de Vos et al. 1992). It has been predicted that dimerization of the intracellular domain of the receptor occurs as well, an association that may generate a site of interaction with intracellular substrates or effector proteins, resulting in signal transduction to the cell nucleus (de Vos et al. 1992).

Direct evidence implicating the GHR in LS includes the absence of specific binding of ^{125}I-labeled GH to liver microsome pellets from LS patients (Eshet et al. 1984) and decreased activity of the GH-binding protein (GHBP) (Daughaday and Trivedi 1987), a circulating proteolytic cleavage product of the receptor (Trivedi and Daughaday 1988), which has an amino acid sequence identical to the extracellular domain (Leung et al. 1987). Five GHR gene mutations, each involving the extracellular domain of the receptor, have been reported in LS patients. The mutations include a complex deletion of three noncontiguous exons in six families (Godowski et al. 1989; Meacham et al. 1992); R43X, a recurrent mutation in two Mediterranean families; C38X in a northern European family (Amselem et al. 1991); and F96S in a Tunisian family (Amselem et al. 1989). In addition, 37 of 38 cases of LS in southern Ecuador are caused by E180splice, a point mutation that generates a new and efficiently used 5′ splice consensus sequence within exon 6 of the GHR gene (Berg et al. 1992). Splicing occurs virtually exclusively at this new splice site, while the intact, normal 5′ splice site 24 nucleotides downstream is not used, causing the predicted deletion of eight amino acids from the GHR protein (Berg et al. 1992).

It has been proposed that the Ecuadoreans affected by the E180splice mutation may descend from Spanish Jews who converted to Christianity during the Inquisition and then emigrated to South America (Rosenbloom et al., in press). If so, the E180splice mutation may be present among other affected individuals of Spanish or Jewish origin. In order to investigate this hypothesis and to further study the molecular basis of LS, we searched for the mutations causing LS in seven additional unrelated patients from Spain, South Africa, the United States, Brazil, and Ecuador. By scanning these patients' GHR genes by denaturing gradient gel electrophoresis (DGGE) (Myers et al. 1987) and by sequencing specific exons likely to have mutations, we have found that LS is caused by many different GHR mutations that arose independently in different parts of the world.

Patients and Methods

Clinical and Biochemical Evaluations

Seven unrelated individuals with LS from Spain, the United States, South Africa, Brazil, and Ecuador were studied. The diagnoses of LS were based on typical clinical and biochemical findings, which are summarized below. The units for all measurements are nanograms per milliliter, unless otherwise specified. Normal ranges are given in parentheses.

Patients 1 and 2 are from Spain, near the northern and southern borders with Portugal. Patient 1 is the product of a first-cousin mating, while patient 2 was born to unrelated parents. Both are female. At ages 18 and 21 years they had, respectively, heights of 115 cm and 115.5 cm (−8.5 SD and −8.4 SD, respectively), basal GH measurements of 50 and 53, IGF-I levels of 49.3 and 16.9 (normal 750 ± 80), insulin-like growth factor binding protein-3 (IGFBP-3) levels of 1.32 ng/μl and 0.27 ng/μl (normal 3.8 ± 0.1), IGFBP-1 levels of 9.1 and 43.0 (10 times and 40 times normal), and GHBP activities of 2.4% and 3.4% of normal.

Patients 3 and 6 are black males from Cincinnati and are not known to be consanguineous. At ages 5 and 13½ years they had, respectively, heights of 81.8 cm and 97 cm (−4.4 SD and −8.9 SD) and random GH levels of 69 and 22.8. Basal IGF-I was undetectable, with no rise in IGF-I with exogenous GH treatment for 4 d, and GHBP activity was undetectable in both cases.

Patient 4 is a South African black female, age 5½ years, born to unrelated parents. Height was 74.4 cm (−8.1 SD), random GH level was 34, IGF-I level was less than 10 (normal 70–288), IGF-II level was 16 (normal 334–642), IGFBP3 level was 0.11 (normal 1.5–3.4), and GHBP activity was 5.2% of normal.

Patient 5, previously reported as CP (Saldanha and Toledo 1981), is a Brazilian male, the product of a first-cousin mating. At age 13 years, height was 87.5 cm (−8.3 SD), GH level was 18, and IGF-1 level was below 3 units/ml. The IGF-1 level remained unchanged with human GH treatment. GHBP activity was not measured.

Patient 7, reported previously (as patient 19 in Rosenbloom et al. 1990), is from Ecuador and is the product of a first-cousin mating. We had previously found that she does not carry the splice mutation that is prevalent in Ecuador (Berg et al. 1992). At age 17 years, height was 121.8 cm (−7.0 SD). Basal GH level (at age 27 mo) was 160. IGF-I level was 43, IGF-II level was 228 (normal 484 ± 33), IGFBP-3 activity was 58% of normal, and GHBP activity was 30% of normal (Rosenbloom et al. 1990).

DNA Preparation and Amplification

Genomic DNA was extracted from fresh or transformed leukocytes or fibroblasts by standard procedures. PCR (Saiki et al. 1988) amplification of all nine coding exons and intron-exon boundaries of the GHR gene (Godowski et al. 1989) from genomic DNA was performed using primers containing GC-clamps (Sheffield et al. 1989), under conditions described elsewhere (Berg et al. 1992), except that the following primers (Amselem et al. 1991) and conditions were used to amplify exon 7: 5′CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCGTAGTGTTCATTGGCATTGAG3′ and 5′ACAAAAGCCAGGTTAGCTAC3′, 1.5 mM $MgCl_2$, annealing temperature 55°C. The 5′ primer contains a 40-nucleotide GC-clamp (Sheffield et al. 1989). When no samples from parents of probands were available for study, 25 μl of the proband's PCR products were sometimes mixed with 25 μl of normal control PCR products and subjected to an additional five cycles of PCR either with or without the addition of 5 units of *Taq* DNA polymerase (Perkin Elmer Cetus). This allowed the formation of heteroduplexes that have significantly altered migration in denaturing gradient gels. Amplification of intron 9 for haplotype analysis was performed using primers P4 and P5 and conditions as described elsewhere (Amselem et al. 1989).

DGGE

PCR products encompassing the entire coding sequence and all intron-exon boundaries of the GHR gene except those of exon 3 were analyzed by parallel DGGE using reagents and equipment as described elsewhere (Myers et al. 1987). DGGE separates DNA fragments according to their melting properties in a polyacrylamide gel containing a gradient of the DNA denaturants, urea and formamide, and maintained at 60°C. The addition of the high-temperature-melting GC-clamp to one end of the fragments being analyzed increases the sensitivity of DGGE such that nearly all single-base mutations can be detected in appropriately sized molecules (Sheffield et al. 1989). Melting characteristics of the fragments were predicted using the MELT program (Lerman and Silverstein 1987), which was provided by R. M. Myers. The ranges of denaturant concentrations and electrophoresis times used are available on request. Gels were stained in ethidium bromide and were photographed with UV transillumination.

DNA Sequencing

Exons were selected for sequencing on the basis of positive results on DGGE. In addition, intron 9 of patient 7 was sequenced for haplotype analysis. Double-stranded PCR products were subjected to electrophoresis in 1% low-melting-temperature agarose gels. The appropriate band was excised, and the fragment was purified using the Magic™ PCR Preps DNA Purification System (Promega). The fragment then was (*a*) sequenced directly by using the CircumVent™ Thermal Cycle Sequencing Kit (New England Biolabs) with ^{35}S-dATP incorporation and (*b*) subcloned by using the TA Cloning System (Invitrogen) and then sequenced using the Sequenase Version 2.0 Sequencing System (United States Biochemical Corporation).

Results

DGGE

All nine GHR coding exons were screened for mutations by using DGGE. For each patient, one exon with abnormal migration on DGGE was identified. DGGE screening results of exons 4 and 7 from patients 1–5 are shown in figure 1. Patients 6 and 7 had abnormal results on screening of exons 7 and 4, respectively. A previously described polymorphism at codon 168 in exon 6 (Leung et al. 1987) was also detected by DGGE.

Mutations

Fragments with abnormal results on DGGE were sequenced directly from both strands and from subclones. Six different mutations in GHR alleles of these seven individuals were identified (fig. 2). Five of these have not been reported previously.

The Ecuadorean, patient 7, is homozygous for R43X (fig. 2*a*). This is a nonsense mutation, involving a CpG dinucleotide, that has also been reported in two Mediterranean families (Amselem et al. 1991).

The dinucleotide deletion 46delTT was detected in exon 4 of patients 1 and 2, who are from Spain (fig. 2*b*). It causes a frameshift leading to an in-frame stop codon five codons downstream. Patient 1, whose parents are first cousins, is homozygous for this mutation, while

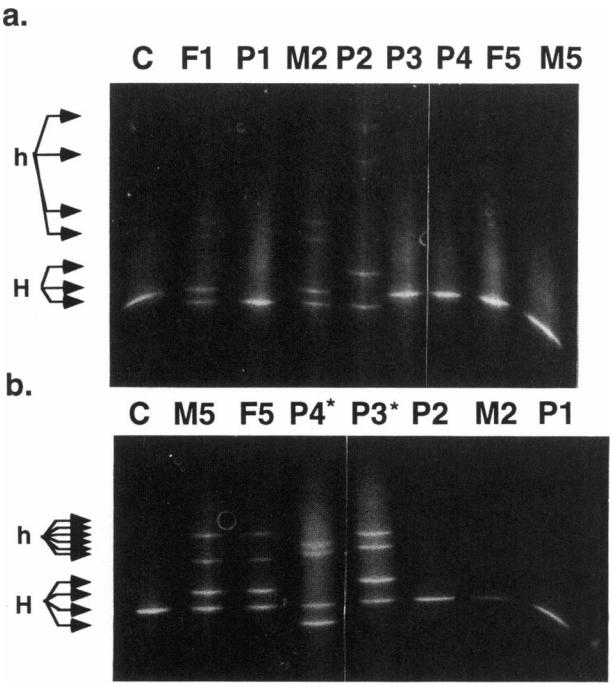

Figure 1 DGGE of exons 4 and 7. Patient lanes are indicated by a "P" and the patient's number; lanes for the respective mother and father are indicated by "M" and "F," respectively; and the lane for a normal control is indicated by "C." Arrows point to homoduplex (H) and heteroduplex (h) bands. Homoduplexes consist of two absolutely complementary DNA strands, while heteroduplexes consist of mismatched complementary strands that paired during PCR of a heterozygous sample. Heteroduplexes have a relatively low melting temperature, because of the mismatch, and therefore have reduced migration in the denaturing gradient gel. Directions of migration and of denaturing gradient are top to bottom. The downward curving of the bands toward the sides of the gels is due to decreased denaturant concentration in the periphery of the gel. *a*, Exon 4. The middle H arrow points to the wild-type homoduplex band, which is the only band present in C. The lower and upper homoduplex bands contain DNA with sequence differences that change the melting behavior. The presence of both normal and lower homoduplexes in the F1 and M2 lanes indicates that these individuals are heterozygous for the normal allele and an allele with a sequence change that increases the melting temperature of the fragment. The P1 lane contains only the lower abnormal homoduplex, indicating probable homozygosity for an exon 4 mutation. The P2 lane has no normal homoduplex band and contains two abnormal homoduplexes, indicating that patient 2 may be a compound heterozygote for two different mutations in exon 4, one of which patient 1 appears to be homozygous for. Only the normal homoduplex band is seen in the P3, P4, M5, and F5 lanes, indicating that these individuals are unlikely to have mutations in exon 4. *b*, Exon 7. Four different homoduplex bands and six different heteroduplex bands are present. The normal homoduplex band is identified in lane C. The parents of patient 5 (M5 and F5) are heterozygous, with the same abnormal homoduplex and heteroduplex bands present. In lanes P4* and P3*, the patient's PCR products were mixed with normal control PCR products and were denatured and renatured prior to being loaded onto the gel. Differently migrating abnormal homoduplex and heteroduplex bands are present, indicating that these patients have heterogeneous sequence changes in exon 7. Only the normal homoduplex is seen in lanes P2, M2, and P1.

patient 2 is a compound heterozygote for this mutation and the 71+1 G-to-A mutation described below.

The Brazilian, patient 5, is homozygous for 189–1 G to T, a substitution of T for G at the −1 position of the 3′ splice consensus sequence of intron 6 (fig. 2*c*). This change alters an absolutely conserved position in the consensus sequence, the second G in Chambon's GT-AG rule (Breathnach and Chambon 1981).

Patients 3 and 6, two black individuals from Cincinnati who are not known to be related, carry a substitution of T for C in the first position of codon 217, generating a stop codon in exon 7 of the GHR (fig. 2*d*). Homozygosity for R217X, versus the unlikely possibility of hemizygosity, was not confirmed in these patients, because of the unavailability of parental DNA.

The South African black, patient 4, has a dinucleotide deletion involving codon 230 in exon 7. On the basis of the normal (CTATG) and mutant (CTG) sequences, either of two T's could be deleted, hence the designation 230 del TA or AT (fig. 2*e*). The deletion causes a frameshift and an in-frame stop codon 4 codons downstream. Again, homozygosity, versus the less likely possibility of hemizygosity, was not confirmed, because of the unavailability of parental DNA.

A substitution of A for G at the +1 nucleotide of the 5′ splice consensus sequence of intron 4, 71+1 G to A (fig. 2*f*), was found in Spanish patient 2 who was a compound heterozygote for this and the 46 del TT mutation. G at this position, the first G of Chambon's GT-AG rule (Breathnach and Chambon 1981), is absolutely conserved among 5′ splice consensus sequences in organisms ranging from viruses to humans (Mount 1982).

Haplotype Analysis

GHR gene frameworks 1–6 have been characterized on the basis of the association of six sequence polymorphisms within intron 9 (Amselem et al. 1989). The R43X mutation found in patient 7 was reported previously in association with framework 2 and 3 haplotypes in two Mediterranean families (Amselem et al. 1991). Analysis of intron 9 sequence from patient 7 indicated that R43X in this patient is present on a framework 1 haplotype of the GHR gene.

Discussion

Using DGGE to narrow sequencing efforts to particular exons likely to have mutations, we have identified six mutations (two dinucleotide deletions and four

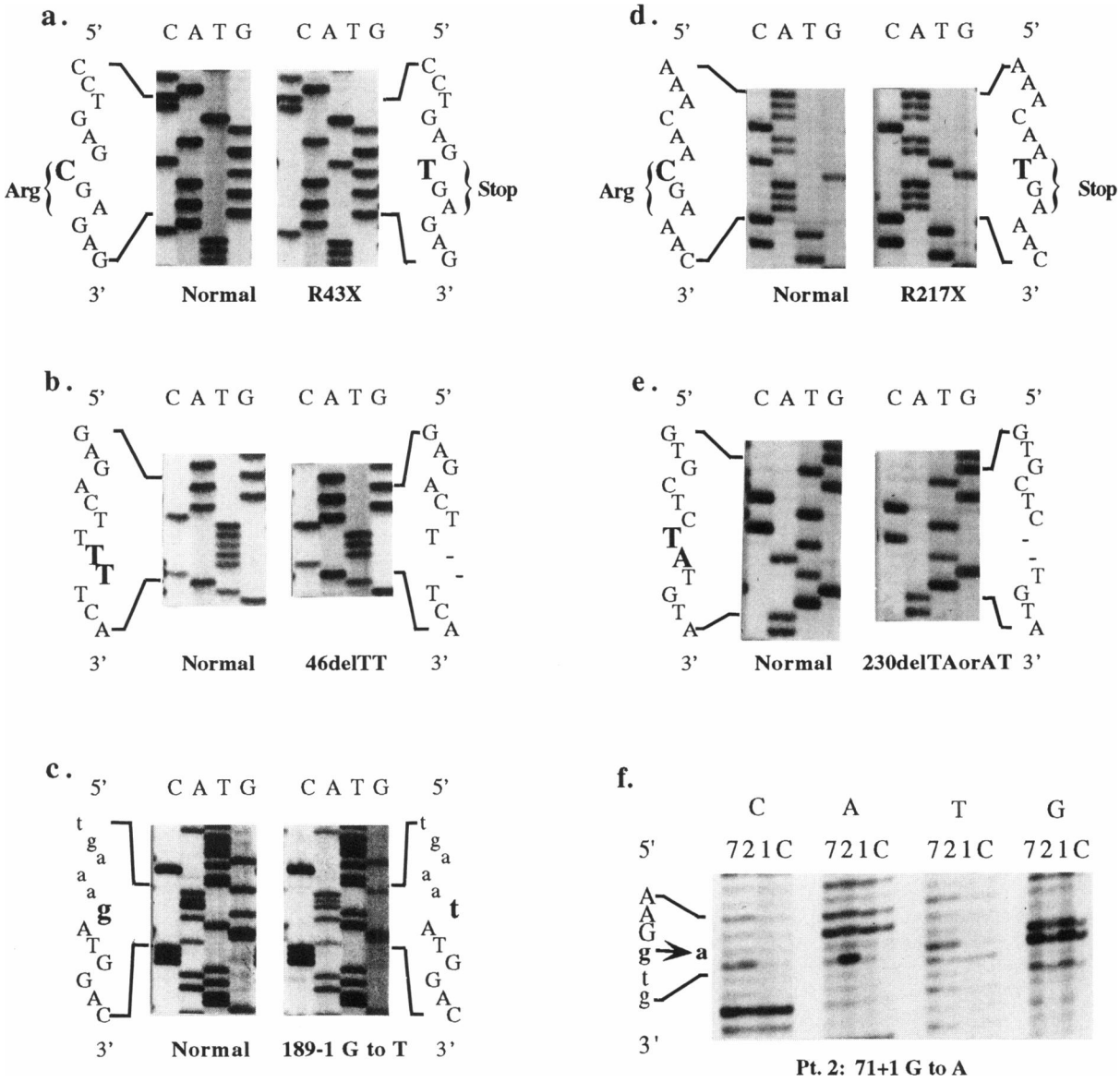

Figure 2 Mutations in exons 4 and 7. Sequences in panels *a–e* were obtained using cloned PCR products as template DNA. Results in panel *f* are from direct sequencing of PCR products. Panels *a*, *b*, and *f* involve exon 4; and panels *c*, *d*, and *e* involve exon 7. Exon sequence is indicated in uppercase letters, and intron sequence is indicated in lowercase letters. *a*, Patient 7, showing a C-to-T transition at a CpG dinucleotide in codon 43, changing an arginine to a stop codon. *b*, Patients 1 and 2, showing deletion of two T's from a series of five T's in codons 45–47. (On direct sequencing, patient 2 was heterozygous for this frameshift mutation). *c*, Patient 5, showing a G-to-T transversion at the −1 position of the intron 6 3′ splice junction. *d*, Patients 3 and 6, showing a C-to-T transition at a CpG dinucleotide in codon 217, changing an arginine to a stop codon. *e*, Patient 4, showing a deletion of either a TA or an AT dinucleotide from codon 230, resulting in a frameshift. *f*, C, A, T, and G reactions from patients 7, 2, 1, and a normal control (C), loaded side by side. Patient 2 is heterozygous for a G-to-A transition, which occurs at position +1 of the intron 4 5′ splice junction, 71+1 G to A.

point mutations) in the GHR genes of seven patients with LS. Each mutation is predicted to have a major deleterious effect on the GHR. Four of the mutations create in-frame stop codons in the extracellular domain, with no translation of the downstream transmembrane or intracellular domains expected. The other two mutations change absolutely conserved nucleotides in 5′ or 3′ splice consensus sequences. Similar splice mutations cause exon skipping (Cole et al. 1990; Kuivaniemi et al. 1990; Lee et al. 1991; Vasan et al.

Table 1

GHR Mutations in Laron Syndrome

Reference	Ethnic Origin	Consanguinity	Mutation
Godowski et al. 1989	Jewish-Iraqi	+	Deletion of exons 3, 5, and 6
Meacham et al. 1992	Iraq, Iran, Yemen, Turkey, Morocco	?	Deletion of exons 3, 5, and 6[a]
Amselem et al. 1989	Tunisia	+	F96S
Amselem et al. 1991	Mediterranean	+	R43X
	Northern Europe	+	C38X
Berg et al. 1992	Ecuador	Inbred population	E180splice
Present report	Afro-American	−	R217X
	Brazil	+	189-1 G to T
	South African	−	230 del TA or AT
	Spain	+	46 del TT
	Spain	−	46 del TT and 71+1 G to A[b]
	Ecuador	+	R43X

[a] One individual is a compound heterozygote with the exon 3, 5, 6 deletion on one chromosome and an unidentified mutation on the other.
[b] Compound heterozygote.

1991; Andrews and Markert 1992) or use of cryptic splice sites (Orkin et al. 1982; Chiodo et al. 1992) in other disorders.

With regard to the mutations' mechanisms of origin, the dinucleotide deletions both occur in regions of direct repeats (TTTTT and TAT), such as those in the immediate vicinity of 59 of 60 short (<20 bp) gene deletions analyzed (Krawczak and Cooper 1991). The exact role that direct repeats play in the genesis of deletions is uncertain. The R217X and R43X mutations involve CG-to-TG transitions, changes that are probably associated with hypermutability of the dinucleotide CpG (Duncan and Miller 1980).

The data presented here and in other reports (summarized in table 1) indicate that a founder effect is likely in Ecuador, where a single mutation accounts for 97% of LS alleles, and also in Jewish patients from the Middle East and northern Africa, where a complex deletion accounts for a significant fraction of mutant alleles. No mutations have been reported in any of the Israeli Jewish patients who do not carry the complex deletion.

Aside from the Jewish and Ecuadorean populations, LS occurs sporadically throughout the world. These sporadic cases are due to mutations that tend to be unique to particular families: C38X in a northern European family (Amselem et al. 1991), F96S in a Tunisian family (Amselem et al. 1989), 46delTT in two Spanish families, 71+1 G to A in a Spanish patient, 189–1 G to T in a Brazilian family, R217X in two black families from Cincinnati, and 230 del TA or AT in a black South African family (present report).

The only mutation found in patients from different geographic regions is R43X, which was previously reported in two Mediterranean families (Amselem et al. 1991). Recurrence of the mutation, rather than identity by descent, was supported by the association of the mutation with different GHR haplotypes (frameworks 2 and 3) in the two families (Amselem et al. 1991). We have now identified R43X in the single Ecuadorean patient known not to carry the predominant Ecuadorean splice mutation (Berg et al. 1992). R43X in this patient is associated with framework 1, supporting a separate mutational event, consistent with the fact that this mutation is a C-to-T transition at a CpG dinucleotide hot spot (Duncan and Miller 1980).

The clinical and biochemical phenotypes of the LS patients with described genetic defects are remarkably similar (Saldanha and Toledo 1981; Amselem et al. 1989; Godowski et al. 1989; Rosenbloom et al. 1990; Amselem et al. 1991). Nine of the 10 reported LS mutations cause gross structural changes in the GHR, such as the deletion of multiple exons, frameshifts, altered splicing, and premature termination. One would expect a complete absence of functional protein with each of these mutations and, therefore, a similar phenotype for each. The remaining mutation, the F96S amino acid substitution, interferes with GHR intracellular transport to the cell membrane, creating a virtual absence of protein in the appropriate cellular location (Duquesnoy et al. 1991).

The types and locations of all the GHR gene mutations reported in LS are summarized in figure 3. No

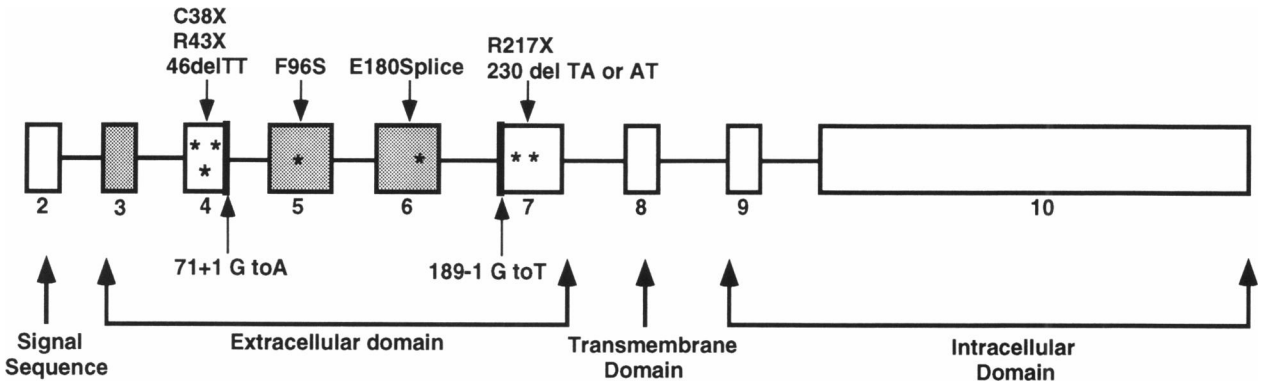

Figure 3 Types and locations of the 10 known GHR mutations. Boxes 2–10 indicate coding exons, separated by lines representing introns. Asterisks (*) indicate the sites of the mutations identified within exons. Thicker bars at intron-exon boundaries indicate the splice-junction mutations. The darkened exons, 3, 5, and 6, are involved in a complex deletion in LS individuals. Mutations have been found in each of the exons encoding the extracellular domain but not in the transmembrane or intracellular domains.

mutations have been identified yet in the exons encoding the intracellular domain of the GHR. Continued molecular analyses of patients with LS may allow the identification of mutations in the intracellular domain of the GHR or in molecules further downstream in the GH pathway, possibly yielding clues regarding unknown aspects of GH action.

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