

## BRIEF REPORT: SHORT STATURE CAUSED BY A MUTANT GROWTH HORMONE

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**T**HE causes of growth hormone–dependent short stature are primary pituitary disease, pituitary deficiency due to hypothalamic dysfunction, and, less often, insensitivity to growth hormone. The prototypical syndrome of growth hormone insensitivity is Laron-type dwarfism, which is characterized by absent or defective growth hormone receptors. Kowarski et al. described two children with growth retardation resulting from biologically inactive growth hormone<sup>1</sup>; additional cases were reported subsequently.<sup>2-7</sup> This disorder is characterized by high serum concentrations of immunoreactive growth hormone, low serum concentrations of insulin-like growth factor I (IGF-I), and increases in both serum IGF-I and linear growth after the administration of exogenous growth hormone. The molecular basis of the disorder is unknown.

In this report we describe a child with short stature and a mutant growth hormone caused by a single missense mutation in the growth hormone gene. In this child the growth hormone not only cannot activate the growth hormone receptor but also inhibits the action of wild-type growth hormone because of its greater affinity for growth hormone–binding protein and growth hormone receptor.

### CASE REPORT

The proband was a boy who weighed 2250 g and was 39 cm long at birth after 41 weeks of gestation. His parents were not related. At the age of 4.9 years, he was 81.7 cm tall (6.1 SD below the mean for age and sex) and had a bone age of 2 years. His body proportions were normal except for a prominent forehead and a saddle nose. The IGF-I concentration was 34 ng per milliliter (normal, 35 to 293). Basal serum growth hormone concentrations ranged from 7.0 to 14.0 ng per milliliter, and peak concentrations after insulin-induced hypoglycemia, arginine administration, and levodopa administration were 38.0, 15.0, and 35.0 ng per milliliter, respectively. Nocturnal urinary growth hormone excretion ranged from 58.8 to 76.7 pg per milligram of creatinine (normal, 7.1 to 41.1). Serum IGF-I concentrations were unchanged by three days of daily subcutaneous injections of 0.1 unit of recombinant human growth hormone per kilogram of body weight (0.035 mg per kilogram). During prolonged treatment with growth hormone (0.18 mg per kilogram per week subcutaneously, given in three divided doses), the serum IGF-I concentration was 200 ng per milliliter and the rate of linear growth increased to 6.0 cm per year (as compared with a rate of 3.9

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Table 1. Clinical Characteristics of the Proband and His Family.\*

CHARACTERISTIC	FATHER	MOTHER	ELDER SISTER	PROBAND	YOUNGER SISTER
Age (yr)	37	33	7	5.6	2.7
Height (cm)	168.7	162.2	127.1	84.8	88.4
SD†	-0.2	1.2	1.7	-6.1	-0.5
Serum growth hormone (ng/ml)					
Basal	1.4	0.2	1.6	7.0	0.2
Peak after insulin-tolerance test	23.7	NT	NT	38.0	NT
Bioactivity‡	1.0±0.2	0.8±0.4	1.2±0.3	0.6±0.2	1.0±0.5
Serum IGF-I (ng/ml)	140	170	160	38	150
Normal	100-315	79-383	86-460	35-293	31-191
Serum IGF-binding protein 3 (µg/ml)	1.9	1.8	3.4	1.6	1.9
Normal	3.4±1.1	3.3±1.0	2.4±0.8	2.4±0.8	2.0±0.6
Serum growth hormone– binding protein (pmol/liter)§	107	194	314	70	136

\*Plus–minus values are means ±SE. NT denotes not tested.

†Number of standard deviations above or below the mean for age and sex.

‡Bioactivity is expressed as the ratio of bioactivity to immunoradiometric activity. The values are the means ±SE of three or more samples. The mean value in 30 normal subjects was 1.0±0.2.

§The normal value is 222±115 pmol per liter in men and 180±92 pmol per liter in women.

cm per year before treatment). The characteristics of the patient and his family are shown in Table 1.

### METHODS

#### Hormone Assays

Serum immunoreactive growth hormone was measured with an immunoradiometric assay kit (Pharmacia, Uppsala, Sweden), and serum bioactive growth hormone was measured with the use of Nb2 rat-lymphoma cells as described previously.<sup>8</sup> In this bioassay, rabbit antiserum to human prolactin (NIDDK-anti-hPRL-IC5; National Institutes of Health, Bethesda, Md.) was added at a dilution of 1:100,000 to neutralize the growth-stimulating action of prolactin. Serum IGF-I and insulin-like growth factor (IGF)–binding protein 3 were measured by radioimmunoassays.<sup>9,10</sup> Serum concentrations of growth hormone–binding protein were determined with use of an assay, as described previously.<sup>11</sup>

#### Isoelectric Focusing

Isoelectric focusing was performed as described previously.<sup>12</sup> Serum samples (200 to 300 µl) were electrofocused in a buffer containing 1 percent hydroxypropyl methylcellulose and 4 percent ampholine (pH gradient, 3.5 to 8.0) at 200 V for 12 hours and then at 500 V for 12 hours. The fractions were collected and assayed for immunoreactive growth hormone. Pooled serum samples from 10 normal subjects were used as the control.

#### Genetic Analysis

Genomic DNA was isolated from peripheral-blood leukocytes<sup>13</sup> and amplified by the polymerase chain reaction (PCR) with three pairs of oligonucleotide primers (F3 and GAD, GSD and GAE, and GHS1 and GAD) (Fig. 1 and Table 2). The PCR amplification with primer pairs F3 and GAD and GHS1 and GAD involved an initial period of denaturation for three minutes at 92°C, followed by 35 cycles consisting of one minute of denaturation at 92°C, two minutes of annealing at 60°C, two minutes of extension at 72°C, and a final period of extension at 72°C for seven minutes. The PCR amplification with the primer pair GSD and GAE involved 35 cycles consisting of one minute of denaturation at 92°C, one minute of annealing at 68°C, and one minute of extension at 72°C. The amplification products were extracted and subcloned into pBluescript SK(+) phagemid (Stratagene, La

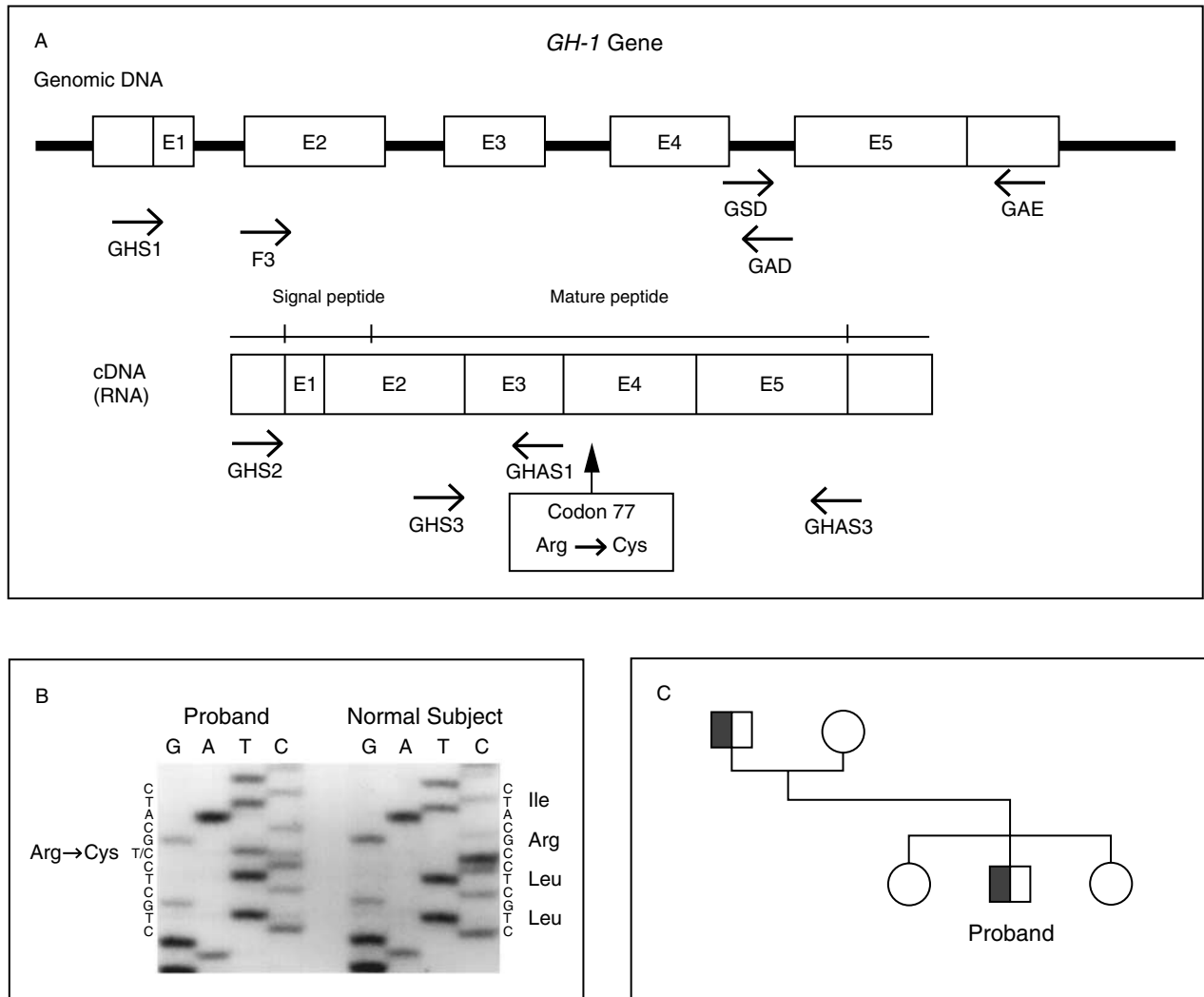


Figure 1. Structure of the *GH-1* Gene and the Primers Used for PCR Amplification (Panel A), the Sequence of the *GH-1* Gene (Panel B), and the Proband's Pedigree and Genotype (Panel C).

In Panel A, the five exons are indicated by boxes (E1, E2, E3, E4, and E5) and the forward and reverse PCR primers are indicated by arrows. The location of the mutation at codon 77 (substitution of cysteine for arginine) is indicated. In Panel B, the heterozygous mutation at codon 77 that results in the substitution of cysteine for arginine is indicated. This mutation was confirmed by direct sequencing analysis in both genomic DNA and RNA. Panel C shows the pedigree. Sequence analysis of the *GH-1* gene was performed in all family members. Squares denote male family members, circles female family members, and the half-solid symbols those heterozygous for the *GH-1* mutation.

Jolla, Calif.) or pT7 blue vector (Novagen, Madison, Wis.) and sequenced with a DNA sequencer (model 373A, Perkin-Elmer, Applied Biosystems, Foster, Calif.). Once a mutation was identified, direct sequencing was performed with a double-stranded DNA cycle-sequencing kit (GIBCO BRL, Grand Island, N.Y.).

#### RNA Analysis

Lymphocytes were separated by mono-poly resolving medium Ficoll-Hypaque density-gradient centrifugation (Flow Laboratories, Costa Mesa, Calif.), and total RNA was isolated as described previously.<sup>14</sup> Then, complementary DNA (cDNA) was synthesized with 1  $\mu$ g of total RNA.<sup>15</sup> The cDNA was used for PCR to amplify cDNA for the growth hormone gene *GH-1*. Two pairs of oligonucleotide primers were used for PCR: GHS2 and GHAS1 and GHS3 and GHAS3 (Fig. 1). The PCR amplification with the primer pair GHS2 and GHAS1 involved an initial period of denaturation for 3 minutes

at 92°C, followed by 40 cycles consisting of 1 minute of denaturation at 92°C, 1.5 minutes of annealing at 68°C, 1.5 minutes of extension at 72°C, and a final period of extension at 72°C for 7 minutes. The PCR amplification with the primer pair GHS3 and GHAS3 involved 40 cycles consisting of 1 minute of denaturation at 92°C, 1 minute of annealing at 60°C, and 1.5 minutes of extension at 72°C. The amplified products were analyzed as described in the preceding section.

#### cDNA Constructs of Wild-Type and Mutant Growth Hormone

Growth hormone cDNA was amplified by PCR with the use of a cDNA library prepared from human growth hormone-producing pituitary adenoma cells, and the accuracy of the structure of growth hormone cDNA was confirmed by sequencing. The oligonucleotide primers used for PCR were GHS1 and 5'TAAGAAATTCGAGGG-GTCCACAGGGATGCCACCCG3' (an antisense primer). The PCR

conditions consisted of an initial period of denaturation at 92°C for 3 minutes, followed by 35 cycles consisting of 1 minute of denaturation at 92°C, 1.5 minutes of annealing at 48°C, 1.5 minutes of extension at 72°C, and a final period of extension at 72°C for 7 minutes. The mutant growth hormone cDNA was constructed with a Transformer mutagenesis kit (Clontech, Palo Alto, Calif.). To remove the signal sequence of growth hormone cDNA, PCR amplification was performed with a sense primer (5'GCGGATCCTTCCCAACCAT-TCCCTTATC3') that includes an artificial *Bam*HI site and GHAS1 as an antisense primer.

### Characterization of the Functional Properties of Wild-Type and Mutant Growth Hormone

Wild-type and mutant growth hormone cDNA was subcloned into a *Bam*HI-*Eco*RI site in the pGEX-KG plasmid vector, which was then transformed into the *Escherichia coli* strain DH5 $\alpha$ . Wild-type and mutant growth hormone was expressed and purified with a glutathione-S-transferase gene fusion system (Pharmacia). The bioactivity of the expression products was determined, and the products were assayed with a bioassay involving the Nb2 cell line. The Nb2 bioassay was performed in the presence and absence of serum from a patient who had undergone hypophysectomy. Recombinant human growth hormone-binding protein was added to the samples in increments of 10  $\mu$ l, resulting in final concentrations of 0.1, 0.5, or 1.0 nM.

Competitive binding studies with [<sup>125</sup>I]human growth hormone were performed in the human lymphoblast cell line IM-9, which expresses growth hormone receptors, as described previously.<sup>16</sup> Direct binding of wild-type and mutant growth hormone to recombinant human growth hormone-binding protein was determined by immunoprecipitation.

Growth hormone-dependent tyrosine phosphorylation in IM-9 cells was determined as described previously, with modifications.<sup>17</sup> Antiphosphotyrosine monoclonal antibody (RC20, Transduction Laboratories, Lexington, Ky.) was used for both immunoprecipitation and Western blotting. Antibody binding was visualized with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom).

## RESULTS

The bioactivity of the proband's growth hormone was below the normal range (Table 1). Isoelectric focusing of the proband's serum revealed the presence of an abnormal growth hormone peak in addition to a normal peak (Fig. 2A); his father's serum contained only one peak (Fig. 2B), as did serum from unrelated normal subjects (data not shown). We then determined the sequence of the *GH-1* gene in the proband. A heterozygous single-base substitution was identified (Fig. 1B), which resulted in the substitution of cysteine for arginine at codon 77. The genotypes of the proband and his family are shown in Fig. 1C.

Table 2. Sequences of the Primers Used to Amplify the Growth Hormone Gene.

PRIMER	SEQUENCE
GHS1	5'GGACAGCTCACCTAGTGCA3'
F3	5'TATGAATTCCTCTGCCTGCCCTGG-CTTCAAGAG3'
GAD	5'CTAACACAGTCTCTCAAAGT3'
GSD	5'ACTTTGAGAGACTGTGTTAG3'
GAE	5'TGGAGTGGCAACTCCAGGG3'
GHS2	5'TGGACGCTCACCTAGTGCA3'
GHAS1	5'GGATTTCTGTTGTTCCT3'
GHS3	5'CTAGAAGCCACAAGCTGCCCT3'
GHAS3	5'CTAGAAGCCACAGCTGCCCT3'

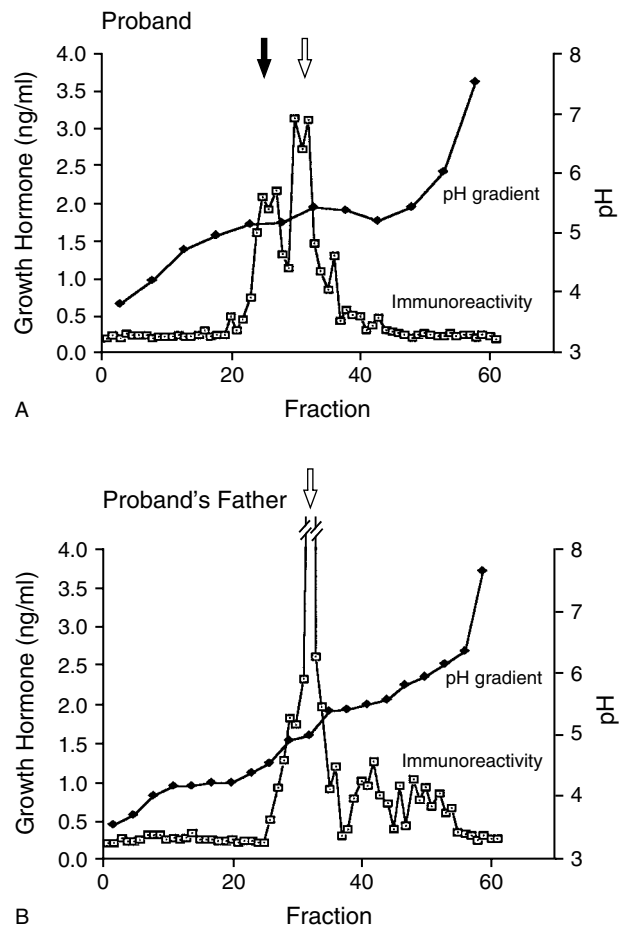


Figure 2. Results of Isoelectric Focusing of Growth Hormone in Serum from the Proband (Panel A) and His Father (Panel B). The serum fractions were pooled separately and assayed for growth hormone immunoreactivity. The pH gradient formed during isoelectric focusing is indicated. The isoelectric point of wild-type growth hormone is pH 4.9 and that of the mutant growth hormone identified in the proband is predicted to be pH 4.7. The peaks for wild-type and mutant growth hormone are indicated by the open and solid arrows, respectively.

To assess whether this mutation was responsible for the inactivity of the proband's growth hormone, the wild-type and mutant growth hormone were expressed as glutathione-S-transferase fusion proteins. Both forms of growth hormone were equally immunoreactive. Although the bioactivity of both proteins was similar when assayed in serum-free medium, the bioactivity of the mutant growth hormone was less than half that of wild-type growth hormone in the presence of serum from a patient who had undergone hypophysectomy, which contained neither growth hormone nor prolactin (data not shown). Because of the possibility of interference by growth hormone-binding protein in the Nb2-bioassay system, recombinant human growth hormone-binding protein was added to the assay medium. The mean ( $\pm$ SE) ratio of bioactivity to immunoreactivity of the mutant growth hormone was significantly reduced to  $45 \pm 5$  percent ( $P = 0.01$ ) and  $22 \pm 8$  percent ( $P = 0.02$ ) of

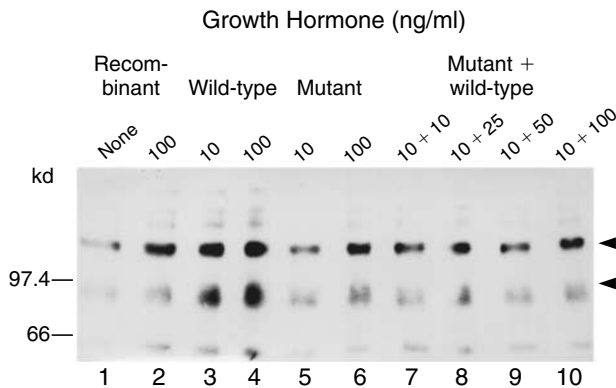


Figure 3. Growth Hormone–Dependent Tyrosine Phosphorylation in IM-9 Cells.

IM-9 cells were incubated without recombinant human growth hormone (lane 1) and with 100 ng of recombinant human growth hormone per milliliter (lane 2); 10 ng and 100 ng of wild-type growth hormone per milliliter (lanes 3 and 4, respectively); 10 ng and 100 ng of mutant growth hormone per milliliter (lanes 5 and 6, respectively); and 10 ng of mutant growth hormone per milliliter and 10 ng, 25 ng, 50 ng, and 100 ng of wild-type growth hormone per milliliter (lanes 7, 8, 9, and 10, respectively) for 15 minutes at 37°C. Detergent lysates of these cells were immunoprecipitated with a phosphotyrosine-specific antibody and analyzed by Western blotting with the same antibody conjugated to horseradish peroxidase. Arrowheads indicate tyrosine-phosphorylated proteins stimulated by growth hormone.

the ratio for wild-type growth hormone in the presence of 0.5 and 1.0 nM recombinant human growth hormone-binding protein, respectively — concentrations similar to those in the serum of normal subjects.

Binding of [<sup>125</sup>I]human growth hormone to human growth hormone receptors in IM-9 cells was inhibited by wild-type and mutant growth hormone in a dose-dependent manner, and the mean concentrations at which binding was reduced by 50 percent were  $0.84 \pm 0.30$  and  $0.86 \pm 0.41$  nM, respectively (mean results of three experiments). Binding of [<sup>125</sup>I]human growth hormone to recombinant human growth hormone-binding protein was also inhibited by the normal and mutant protein in a dose-dependent manner; the 50 percent inhibitory concentration for the mutant protein ( $0.12 \pm 0.02$  nM [mean results of three experiments]) was significantly lower than that for wild-type growth hormone ( $0.68 \pm 0.08$  nM), indicating that the affinity of the mutant growth hormone for growth hormone-binding protein was approximately six times higher than that of wild-type growth hormone.

The mutant growth hormone not only failed to stimulate tyrosine phosphorylation by itself, but it also inhibited the activity of wild-type growth hormone, even when the concentration of wild-type growth hormone was 10 times higher than that of the mutant type (Fig. 3).

#### DISCUSSION

The proband was a boy with severe growth retardation (6.1 SD below the mean for age and sex at the age of 4.9 years) and delayed bone age who had high basal

serum growth hormone concentrations and low IGF-I concentrations and increases in serum growth hormone on provocative testing. Although these findings were consistent with the presence of the growth hormone-insensitivity syndrome,<sup>18</sup> that diagnosis was excluded because somatic linear growth improved during long-term administration of growth hormone. The serum growth hormone in this child was less bioactive than that of normal subjects, and the presence of an abnormal growth hormone molecule in his serum was confirmed by isoelectric focusing.

The patient's abnormal growth hormone resulted from the replacement of arginine by cysteine at codon 77 of the *GH-1* gene. This codon is located in the second  $\alpha$  helix of the growth hormone molecule, behind a site of binding to the growth hormone receptor.<sup>19,20</sup> The substituted cysteine may form a new disulfide bond, changing the charge or conformation of the growth hormone molecule and thereby reducing the bioactivity of the mutant growth hormone.

Dimerization of growth hormone receptors induced by ligand binding and sequential protein phosphorylation in tyrosine residues are crucially important for growth hormone-induced signal transduction.<sup>21-24</sup> The mutant growth hormone not only did not stimulate tyrosine phosphorylation in IM-9 cells but also inhibited the ability of wild-type growth hormone to stimulate tyrosine phosphorylation, thus having a dominant negative action.

Serum growth hormone-binding protein is structurally identical to the extracellular domain of the growth hormone receptor and serves as a growth hormone reservoir in vivo.<sup>25-27</sup> In our patient, the affinity of the mutant growth hormone for growth hormone-binding protein was significantly higher than that of wild-type growth hormone. The binding of growth hormone to growth hormone receptors is believed to proceed sequentially (i.e., first at site 1 and then at site 2).<sup>19</sup> Our findings suggest that the properties of the mutant growth hormone differ from those of wild-type growth hormone with respect to binding affinities at site 1 or 2.

The proband's father was phenotypically normal even though he had the same genetic abnormality as the proband. Isoelectric focusing revealed that the father's serum contained a single growth hormone peak corresponding to wild-type growth hormone. Why the mutant growth hormone gene was not expressed in the father is not known, although genomic imprinting cannot be ruled out.

In conclusion, we found a heterozygous missense mutation in the growth hormone gene of a child with severe growth retardation. As compared with wild-type growth hormone, the mutant growth hormone had a higher affinity for growth hormone-binding protein and was less active in phosphorylating growth hormone signal-transduction molecules. It also inhibited the action of wild-type growth hormone.

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**CORRECTION**

**Short Stature Caused by a Mutant Growth Hormone**

Short Stature Caused by a Mutant Growth Hormone . On page 434, in Table 2, the sequences of the primers GHS1, GHS2, and GHS3 were wrong. The correct sequences, with the nucleotide numbers shown in parentheses, are shown below.

GHS1 (526–555): 5'CTCGAATTCCTGTGGACAGCTCACCTAGCT3'

GHS2 (538–558): 5'TGGACAGCTCACCTAGCTGCA3'

GHS3 (969–988): 5'TTGACACCTACCAGGAGTTT3'