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Severe Short Stature and Endogenous Growth Hormone Resistance in Twin Brothers Without Growth Hormone Gene Mutations

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Growth failure in children with high growth hormone (GH) levels, low insulin-like growth factor 1 (IGF-1) levels, and accelerated linear growth in response to exogenous GH is presumed to result from biologically inactive GH. A molecular diagnosis has only been made in two such patients. We analyzed the presentations and the *GH-1* genes of twin Egyptian brothers with this phenotype. At 8 yr of age, the boys' heights were –4 SD. Their IGF-1 levels were 64 and 60 ng/mL, baseline GH levels were 2.1 and 11.7 mU/L, and growth hormone binding protein levels were normal. Twin B attained a peak GH level of 30.6 mU/L after L-dopa stimulation (Twin A was not tested). After 1 yr of exogenous GH, their growth velocities were >11 cm/year (>97%). Analysis of their *GH-1* exons and introns revealed no mutations, but five polymorphisms were identified that have not been previously reported. The *GH-1* DNA sequence was transfected into human cells and the resulting *GH-1* transcripts were analyzed. Wild-type *GH-1* mRNAs were observed, demonstrating that the polymorphisms do not affect transcript processing. Therefore, although no evidence of *GH-1* gene mutations or abnormal *GH-1* mRNA processing was found, the subjects' excellent response to exogenous GH supports a trial of GH in children with severe short stature, low IGF-1 levels and normal GH responses to stimulation testing.

Key Words: Growth disorders; somatotropin; DNA analysis; biological activity.

Introduction

Production of a bioinactive form of growth hormone was first proposed as an etiology for severe short stature in 1978 by Kowarski et al. *(1)*. These investigators described two patients with severe short stature, slow rates of growth, and very low IGF-1 levels despite normal to elevated baseline and stimulated GH levels. These two unrelated patients showed improved linear growth in response to exogenous GH therapy with a concomitant rise in IGF-1 levels. A few similar cases were subsequently described *(2–5)*. However, it was not until 1996 that a molecular diagnosis was made in a patient with "Kowarski syndrome." The patient was a boy of Japanese descent with growth failure, normal GH, and low IGF-1 levels. He was found to have a single missense mutation in exon 4 of the *GH-1* gene *(6)*. The following year, an unrelated Japanese child with a similar phenotype was described who possessed a different point mutation in exon 4 of the *GH-1* gene *(7)*. Aside from these two patients, a molecular diagnosis has not been made in any other cases of growth failure, due to a lack of responsiveness to endogenous GH, when the patients have demonstrated an excellent response to exogenous GH therapy. We describe twin boys with Kowarski syndrome. Comprehensive molecular analysis of their *GH-1* genes was performed and is described.

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Results

Clinical Response to Therapy

Both boys had significant acceleration of their linear growth in response to exogenous GH. After 1 yr, growth velocities were >97% for age (Fig. 1). After 2 yr of therapy, Twin A's height had improved to –1.7 SD and Twin B's height was –2.0 SD (Table 1). IGF-1 levels also normalized on treatment (Table 2).

Molecular Analysis of the GH-1 *Gene*

Analysis of the *GH-1* gene was performed using genomic DNA from each subject in this study. The PCR was used to amplify the entire *GH-1* gene of each subject, and DNA sequencing was performed to mutations potentially responsible for the disease phenotype. A 2.2 kb DNA fragment containing the *GH-1* gene (five exons, four introns, and 5' and 3' untranslated regions) was amplified from the DNA of each subject. Several DNA sequencing primers were used in order to analyze the entire *GH-1* gene. No mutations were found in the coding sequence of either *GH-1* gene allele of either subject. Twenty-seven polymorphisms

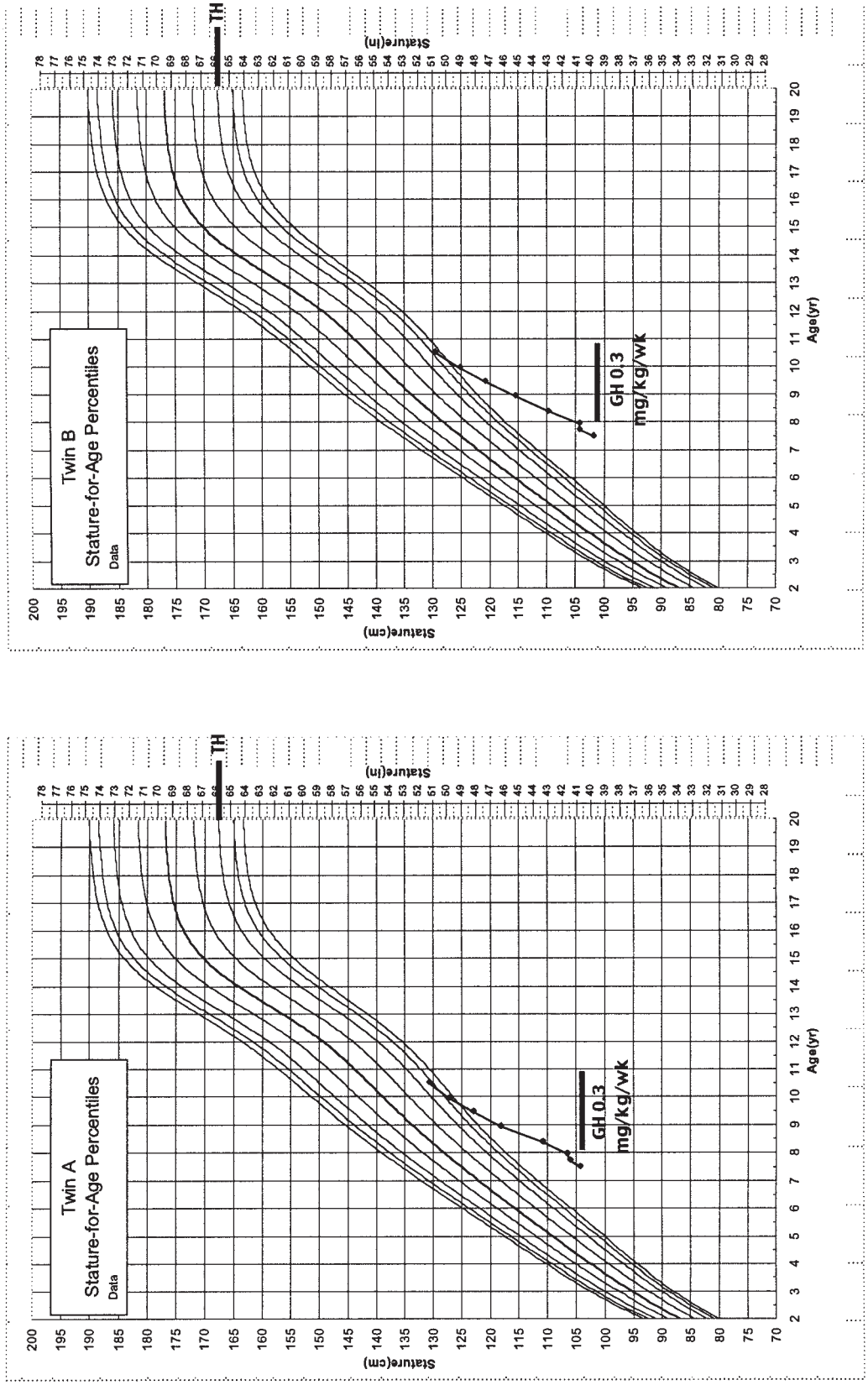

Fig. 1. Growth charts of the subjects demonstrate the acceleration of linear growth in response to exogenous GH therapy. TH = target height.

Table 1
Auxologic Data for the Patients

	Twin A		Twin B	
Age at presentation (yr)	7.5		7.5	
Height at presentation (cm)	106.5	–3.9 SD	104.2	–4.3 SD
Height after 1 yr of GH (cm)	118.2	–2.5 SD	115.4	–3.0 SD
Height after 2 yr of GH (cm)	127.2	–1.7 SD	125.3	–2.0 SD
Growth velocity prior to GH therapy (cm/yr)	4.6	–0.9 SD	4.7	–0.7 SD
Growth velocity after 1 yr of GH therapy (cm/yr)	11.7	+8.4 SD	11.2	+7.8 SD

Table 2
Biochemical Data for the Patients

	Twin A	Twin B	Age adjusted normals	Mother	Father
Baseline GH (mU/L)	11.7	2.1		0.7	0.5
Peak GH-arginine (mU/L)		10.4			
Peak GH-levodopa (mU/L)		30.6			
Acid labile Subunit (mg/L)	7.5	8.2	(adults: 8.6–17.4)	14.1	14.5
IGF-1 prior to GH therapy (ng/mL)	64	60	42–210	127	127
IGF-1 after 30 mo of GH therapy (ng/mL)	208	262	85–435		
IGF-BP3 prior to GH therapy (mg/L)	2.2	2.3	0.95–4.36	2.9	3.6
Growth Hormone Binding Protein on GH therapy (pmol/L)	1143	1233	431–1892		
Karyotype		46XY			

were identified, five of which have not been previously reported (Table 3). Two of these novel polymorphisms occurred in intronic sequences, although they were not found in splice-acceptor or splice-donor sites. The remaining three unreported polymorphisms occurred in either the 5' or 3' untranslated regions.

We next performed in vitro transfection experiments to determine whether any of the identified polymorphisms contributed to the disease phenotype by affecting normal *GH-1* RNA processing. Both identified *GH-1* alleles of the subjects were ligated into mammalian expression vectors, and these DNA plasmids were transfected into human 293T kidney cells. Total RNA was isolated from these cells and cDNA was synthesized in order to characterize the processed *GH-1* transcripts. The PCR then was used to amplify *GH-1* cDNAs and agarose gel electrophoresis was performed to examine the size of the amplified products. The *GH-1* cDNA fragments of each allele of both subjects migrated to the same position as normal controls (Fig. 2). Finally, these cDNAs were isolated and DNA sequencing was performed. Sequencing confirmed that these fragments contained the correct *GH-1* cDNA sequence. These results demonstrate that aberrant *GH-1* gene splicing does not appear to be responsible for the disease phenotype in these subjects.

Discussion

A number of patients with growth failure, normal GH levels, and low IGF-1 levels have had excellent linear growth responses to exogenous GH therapy. Some of these patients had normal GH levels by radioimmunoassay but reduced radioreceptor assay activity *(4,8)*. Using the Nb2 rat lymphoma cell proliferation assay to estimate the activity of endogenous GH in serum, other patients were found to have decreased GH activity *(6–9)*. Based on this evidence, therefore, it was presumed that these patients had biologically inactive GH molecules, also known as the Kowarski syndrome *(1)*. Takahashi et al. *(6,7)* provided evidence that this occurred in two patients due to novel heterozygous mutations in exon 4 of the *GH-1* gene. Both mutations resulted in the production of an abnormal GH molecule that had the ability to bind to the GH receptor (GH-R) but did not result in signal transduction. Additionally, this mutant GH inhibited wild-type GH binding to the GH-R. One other group looked for *GH-1* mutations in three patients with the clinical characteristics of Kowarski syndrome but was unable to identify a mutation in the *GH-1* gene *(9)*.

Although a mutation affecting the coding region of the *GH-1* gene was not detected in our patients, we believe that their phenotype, laboratory characteristics, and response to therapy fit with the clinical description of patients with presumed bioinactive GH or "GH-dependent growth failure" *(4)*. Patients with growth hormone insensitivity syndrome (GHIS) (Laron dwarfs) exhibit growth failure, high GH levels, and low IGF-1 levels, and are unresponsive to exogenous GH therapy. Typically, these patients have low growth hormone binding protein (GHBP) levels as a reflection of the defective GH-R. However, a small number of patients with growth failure, heterozygous *GH-R* mutations, and

Table 3
Polymorphisms in the *GH-1* Genes of the Patients[a]

	Nucleotide	Location	Patients A/B		Acc. No. M13438	Acc. No. J03071
			Allele 1	Allele 2		
1*	409	5' UTR	T	T	A	A
2	440	5' UTR	G	G	T	G
3*	491	5' UTR	G	A	A	A
4	591	Intron 1	G	G	—	G
5	657	Intron 1	T	T	G	T
6	660	Intron 1	G	G	T	G
7	664	Intron 1	C	C	G	C
8	687	Intron 1	G	G	C	G
9	725	Intron 1	TC	TC	CT	TC
10	729	Intron 1	TG	TG	CT	TG
11	732	Intron 1	AG	AG	GA	AG
12*	776	Intron 1	C	T	T	T
13	778	Intron 1	C	C	T	C
14	1049	Intron 2	G	G	—	G
15	1093	Intron 2	—	—	C	—
16	1094	Intron 2	A	A	C	A
17	1127	Intron 2	TG	TG	CT	TG
18	1146	Intron 2	C	C	—	C
19	1157	Intron 2	T	T	A	T
20	1162	Intron 2	C	C	—	C
21	1166	Intron 2	GT	GT	TC	GT
22	1626	Intron 4	—	—	A	—
23	1645	Intron 4	—	—	G	—
24*	1665	Intron 4	A	A	T	T
25	1736	Intron 4	CC	CC	—	CC
26*	2569	3' UTR	G	G	—	—
27*	2600	3' UTR	T	T	C	C

[a]Sequences in the alleles of the twins are compared to two entries in the database. Nucleotide numbers are those of GenBank accession number M13438. Asterisks indicate novel polymorphisms. Dashes indicate a gap at that position. UTR = untranslated region.

low GHBP levels have shown modest responses to GH *(10)*. Recently, numerous cases of GHIS have been reported with normal or even elevated GHBP levels when the *GH-R* mutation affects the transmembrane or cytoplasmic domain *(11–13)*. Thus, the normal GHBP levels of our patients do not exclude a possible mutation in the *GH-R* gene as the etiology of the growth failure. However, the robust response to exogenous GH treatment, which has now been sustained for almost 3 years, is inconsistent with GHIS. The possibility of abnormalities of post GH-R intracellular signaling also exists.

We report five novel polymorphisms in the noncoding sequences of the *GH-1* gene. Importantly, stringent methodological parameters, including performing multiple PCR cloning and sequencing assays using proofreading enzymes, were used to identify and confirm the described polymorphisms. Expression of the amplified gene product by transfection assays revealed that these polymorphisms do not affect the sequence of the *GH-1* transcript. Single-nucleotide polymorphisms (SNPs), which do not result in amino acid changes, in the coding regions of two human genes have been described to affect mRNA structural folding *(14)*. This change in secondary structure of mRNA has been hypothesized to result in abnormalities of splicing, processing, and translation. Nonetheless, because a normal GH molecule was obtained from RNA processing experiments, it is unlikely that an abnormally processed GH molecule accounts for our patients' phenotypes. However, based on the recently noted association of abnormally high levels of insulin secretion observed in obese patients with class I alleles of the variable number of tandem repeats that are linked to a polymorphism near the translational initiation codon of the *insulin* gene *(15)*, it is possible that one of the polymorphisms detected in the *GH-1* gene, or a yet unidentified abnormality in the promoter region, might result in aberrant GH secretion patterns.

Another possible explanation of our patients' growth failure, despite normal GH levels, is that abnormal GH poly-

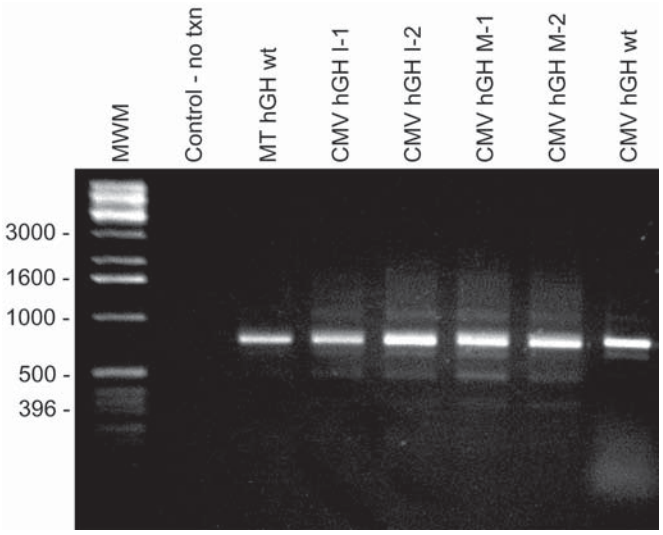

Fig. 2. Molecular analysis of *GH-1* gene splicing. To examine whether the identified polymorphisms affected *GH-1* gene processing, human 293T cells were transfected with expression vectors containing the *GH-1* alleles of each subject (A-1, A-2, B-1, B-2) or wild-type *GH-1* genes (wt) as positive controls. RNA then was isolated from these cells (or from non-transfected cells as a negative control [no txn]) and *GH-1* cDNAs were amplified by PCR. The amplified *GH-1* cDNAs of each subject migrate to positions equivalent to the normal controls. MWM = molecular weight marker; MT = metallothionine promoter; CMV = cytomegalovirus promoter; hGH = human growth hormone.

mers exist in the circulation of these boys. Valenta et al. *(8)* reported a case of a 14-yr-old boy with growth failure and surprisingly normal GH and IGF-1 levels who had abnormally high quantities of very large (85,000-dalton and 45,000-dalton) circulating GH polymers. These polymers were thought to be resistant to conversion to the active monomer form and result in decreased bioactivity with limited growth promoting effects *(16)*. Products of the *GH-1* gene may under normal circumstances undergo acetylation, phosphorylation, deamidation, or glycosylation *(17,18)*. Each of these modified forms of GH have different bioactivity levels. Abnormal posttranslational modifications may occur as a result of one of these newly identified intronic polymorphisms in vivo, but based on our in vitro transfection studies, we currently do not believe that this is the explanation for the decreased GH bioactivity in these boys. We are currently unable to test this possibility as we have inadequate pretreatment serum, and the patients continue on exogenous GH therapy. Alternatively, abnormal posttranslational modification of GH may occur in these boys due to a generalized abnormality in protein modification, for example, abnormal glycosylation patterns, but we would expect a phenotype with multiple other abnormalities in addition to growth failure.

In conclusion, we have identified two boys with growth failure, normal to high GH levels, low IGF-1 levels, and normal *GH-1* genes. They have had an outstanding clinical response to GH therapy. Based on these two patients and a review of the literature, it is apparent that a trial of GH therapy should be undertaken in children with severe short stature, low IGF-1 levels but normal GH responses to stimulation testing. We believe that a rigid policy of treating only children who "fail" conventional GH stimulation testing is flawed.

Patients and Methods

Patients

Identical twin Egyptian boys initially presented to a physician in Egypt at 5 yr of age due to concerns of short stature. Twin A's initial laboratory evaluation revealed normal thyroid hormone and thyroid stimulating hormone levels, a low IGF-1 level (11 ng/mL), a bone age delay of 3.5 yr and a height of –3.7 SD. GH sampling after clonidine administration revealed a baseline of 4.6 ng/mL and a peak of 13.0 ng/mL (1 ng/mL = 2.6 mU/L) with a simultaneous cortisol level of 28 μg/dL (1 μg/dL = 27.6 nmol/L). No therapy was initiated.

At 7.5 yr of age, the twins presented to our institution for reevaluation. Birth history revealed that the boys were born via spontaneous vaginal delivery 1–2 wk preterm after an uncomplicated gestation. Twin A's birthweight was 2025 g and Twin B had a birthweight of 2000 g. Both boys went home 2 d after delivery. Gross motor and dental development were both mildly delayed. The boys did not have any chronic medical conditions and were not taking any medications. They both had experienced mild learning difficulties in school. The family history was negative for growth

problems or consanguinity. Maternal height was 155.7 cm and paternal height was 165.6 cm. Target height was calculated to be 167.1 cm (–1.5 SD).

On physical examination, the boys were dysmorphic in appearance. They had triangular shaped facies with broad frontal regions, protruding, posteriorly rotated ears with simple helices and underdeveloped antihelices. They displayed prominent maxillae and long thin noses. They had fifth finger clinodactyly, hyperextensible joints, and mild hypotonia. Neither boy had café-au-lait macules or body asymmetry. The boys were evaluated by two geneticists who did not believe that their unusual phenotypes were consistent with any recognized syndrome.

Both twins had heights of approximately –4 SD (Table 1). After a period of observation of 6 mo, the growth velocities of both twins were ≤10% for age. GH stimulation testing was then performed on only one of the twins because the family did not have medical insurance. Normal baseline and stimulated GH levels were observed (Table 2). IGF-1 levels were at the lower range of normal. Measurements of the acid labile subunit (ALS), insulin-like growth factor binding protein 3 (IGFBP-3), and GHBP were all normal (Table 2). A trial of exogenous GH therapy was undertaken.

Informed written consent approved by the Institutional Review Board of Indiana University was obtained from both parents and both subjects.

Diagnostic Procedures to Analyze the GH-1 *Gene*

Genomic DNA was extracted from peripheral blood using a QIAamp Blood Maxi Kit (Qiagen, Valencia, CA). The *GH-1* gene of both patients and a normal control was amplified by PCR using 2.5 U Expand High Fidelity DNA polymerase mixture (Roche Biochemical, Indianapolis, IN), 10 mM dATP, dCTP, dGTP, and dTTP, 200 ng genomic DNA, and 10 pmol of the forward 5'-gcataaatgtagcacagaaacagg-3' and reverse 5'-acagccctaggcccaggaattca-3' primers. PCR cycling parameters were as follows: 94°C 2 min, 94°C 10 s, 60°C 10 s, 72°C 2 min for 25 cycles. Three independent PCR experiments were performed, and the amplified *GH-1* genes (2260 base pairs) were analyzed for gross abnormalities on 1% agarose, Tris-borate gels, and, subsequently, ligated into pCRII-TOPO (Invitrogen, Carlsbad, CA). Four PCR products of each of the three independent reactions were sequenced in order to characterize both alleles. DNA sequencing was performed on both strands by automated DNA sequencing using a Perkin Elmer DNA Sequencer (Biochemistry Biotechnology Facility, Indiana University School of Medicine) and the following primers: 5'-gcataaat gtagcacagaaacagg-3', 5'-aacagctcctggagcagggaga-3', 5'-tct gactacaggtctcccccat-3', 5'-tctctaacacagtctctcaaagt-3', and 5'-tttattaggacaaggctggtgg-3'.

Diagnostic Procedures to Analyze GH-1 *Gene Splicing*

DNA fragments representing both *GH-1* alleles from each patient and a normal control were ligated into *Bam*HI/*Eco*RI sites of the pcDNA3 expression vector (Invitrogen). One microgram of each expression vector was transfected into human embryonic kidney 293T cells that were cultured in Dulbecco's modified Eagle medium (Life Technologies, Bethesda, MD) with 10% fetal bovine serum (Irvine Scientific, Irvine, CA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Irvine Scientific); $4.5 \leftrightarrow 10^5$ cells/100 mm dish were transfected with calcium phosphate/DNA precipitates using the CalPhos system (Clontech, Palo Alto, CA). Cells were maintained for 48 h and total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). Complementary DNA was synthesized using Superscript II reverse transcriptase (Life Technologies) and random hexamers. PCR was performed as above with 2 µL of the cDNA reactions and the forward 5'-gtcctgtggacagctc accta-3' and reverse 5'- tttattaggacaaggctggtgg -3' primers. PCR cycling parameters were as follows: 94°C 2 min, 94°C 10 s, 60°C 10 s, 72°C 45 s for 25 cycles. The *GH* cDNAs (767 base pairs) were examined for the presence of aberrant spliced products on 1% agarose, Tris-borate gels, and the fragments were ligated into pCRII-TOPO. The products were completely sequenced as described above.

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