0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(3):980–986

doi: 10.1210/jc.2005-1703

A Novel Deletion in the *GH1* Gene Including the IVS3 Branch Site Responsible for Autosomal Dominant Isolated Growth Hormone Deficiency

Daniela Vivenza, Laura Guazzarotti, Michela Godi, Daniela Frasca, Berardo di Natale, Patricia Momigliano-Richiardi, Gianni Bona, and Mara Giordano

Unit of Pediatrics (D.V., G.B.), Department of Medical Sciences, Eastern Piedmont University, 28100 Novara, Italy; Department of Pediatrics (L.G., D.F., B.d.N.), Luigi Sacco Hospital, University of Milan, 20157 Milan, Italy; and Laboratory of Human Genetics (M.Go., P.M.-R., M.Gi.), Department of Medical Sciences, Eastern Piedmont University and Interdisciplinary Research Center on Autoimmune Diseases, 28100 Novara, Italy

Context: The majority of mutations responsible for isolated GH type II deficiency (IGHD II) lead to dominant negative deleteriously increased levels of the *GH1* exon 3 skipped transcripts.

Objective: The aim of this study was the characterization of the molecular defect causing a familial case of IGHD II.

Patients: A 2-yr-old child and her mother with severe growth failure at diagnosis (−5.8 and −6.9 SD score, respectively) and IGHD were investigated for the presence of *GH1* mutations.

Results: We identified a novel 22-bp deletion in IVS3 (IVS3 del+56–77) removing the putative branch point sequence (BPS). Analysis of patients' lymphocyte mRNA showed an excess exon 3 skipping. The mutated allele transfected into rat pituitary cells produced four differently spliced products: the exon 3 skipped mRNA as the main product and lower amounts of the full-length cDNA and of two novel mRNA aberrant isoforms, one with the first 86 bases of exon 4 deleted and the other lacking the entire exon 4. A mutagenized construct lacking exclusively the 7 bp of the BPS only generated the exon 4 skipped and the full-length isoforms. The presence of the full-length transcript in the absence of the canonical BPS points to an alternative BPS in IVS3.

Conclusion: The IVS3 del+56–77 mutation, causing IGHD II in this family, has two separate effects on mRNA processing: 1) exon 3 skipping, analogous to most described cases of IGHD II, an effect likely caused by the reduction in size of the IVS3, and 2) partial or total exon 4 skipping, as a result of the removal of the BPS. (***J Clin Endocrinol Metab* 91: 980–986, 2006**)

APPROXIMATELY 75% OF the circulating GH is a 191-amino-acid peptide with a molecular mass of 22 kDa. Alternative splicing of the original transcript gives rise to minor amounts of several smaller isoforms. The most represented of these alternative spliced transcripts lacks the first 45 bp of exon 3 through activation of an in-frame cryptic splice site and produces a 20-kDa peptide missing amino acids 32–46 (1). Complete skipping of exon 3, accounting for 1–5% of the total *GH1* transcripts, results in a 17.5-kDa form lacking amino acids 32–71. Two isoforms lacking exons 3–4 and exons 2–4 and encoding 11.3- and 7.4-kDa peptides have also been detected (2).

Mutations in the *GH1* gene have been identified both in type I (autosomal recessive) and type II (autosomal dominant) familial isolated GH deficiency (IGHD) (2, 3). IGHD II is caused mainly by mutations affecting GH mRNA splicing, falling within the first six bases of the IVS3 5′ donor splice site (4–8) or disrupting splicing enhancer elements. The latter includes two mutations in an exon 3 splicing enhancer, ESEm1 + 1G→T (9) and ESEm2 + 5A→G (10), and two mutations in an IVS3 splicing enhancer, ISEm1(IVS3 + 28 G→A) and ISEm2(IVS3Δ28–45) (11, 12). All the reported splicing mutations lead to deleteriously increased levels of the exon 3 skipped transcripts encoding the 17.5-kDa isoform. This isoform lacks the protein linker domain between helix 1 and helix 2 of the mature GH and a cysteine residue, Cys^{53}, involved in the interaction between helix 1 and helix 4 (13). Thus, without the amino acids encoded by the third exon, the molecule cannot fold normally. This altered GH structure exhibits a dominant negative effect on secretion of the 22-kDa isoform. The 17.5-kDa isoform is initially retained in the endoplasmic reticulum, disrupts the Golgi apparatus, impairs both GH and other hormonal trafficking (14), and decreases the intracellular stability of the wild-type GH (15). Moreover, the 17.5-kDa isoform causes a dose-dependent disruption of GH secretory vesicles when expressed in GC cells and transgenic mice (16).

In addition to the above cited splicing mutations, three missense mutations, V110F, P89L, and R183H, are reported (8, 17, 18).

We here describe a novel splicing mutation in an IGHD II family, a 22-bp deletion in the IVS3 of the *GH1* gene encompassing the branch point sequence (BPS) (11). This mutation generates at least three aberrant isoforms: the more abundant exon 3 skipped isoform and two minor isoforms lacking the first 86 nucleotides of exon 4 and the entire exon 4, respectively.

First Published Online December 20, 2005.

Abbreviations: BPS, Branch point sequence; IGHD, isolated GH deficiency; SDS, SD score.

Subjects and Methods

Subjects

The proband, a 2-yr-old girl, came to our observation for severe growth failure at the age of 7 months. She was born by spontaneous delivery after a normal pregnancy at 36 wk gestation with a weight of 2720 g (25th percentile) and a length of 44 cm (3rd percentile). Her phenotype had the IGHD characteristic features: slow but harmonious body development with regular segment proportions, prominent frontal bones, and saddle nose. After biochemical evaluations that excluded organic diseases, two stimulation tests for GH secretion study were performed showing a complete GH deficiency (immunoradiometric assay; DiaSorin, Stillwater, MN) confirmed by the IGF-I dosage (chemiluminescent enzyme-labeled immunometric assay; Medical System, Los Angeles, CA) (Table 1). Analysis of TSH (electrochemiluminescence immunoassay; Roche, Indianapolis, IN) and cortisol (RIA; Adaltis, Montreal, Canada) excluded other anterior pituitary hormone deficiencies (Table 1). The magnetic resonance imaging of the pituitary region performed with narrow scanning and gadolinium injection reported a normal anatomy of the region with the sagittal T1 images showing a mild hypoplasia of the pituitary gland with a height at −2.0 SD score (SDS) for age (21). Therapy with human recombinant GH, started at the age of 9 months at the dose of 0.033 mg/kg·d, yielded a very good response; the growth velocity reached 20 cm/yr (Table 1).

The proband's mother, now 39 yr old, arrived for medical observation for severe growth failure at the age of 4.6 yr. She was born after a normal pregnancy and delivery, but no information is available about her weight and length at birth. Her clinical and hormonal characteristics are shown in Table 1. She was treated, in a noncontinuous way, with human pituitary-derived GH until the age of 16 yr with a final height of 146 cm, which was her target height (mother's height, 148 cm; father's height, 160 cm). After GH suspension at the age of 16, she developed severe obesity. A new test for hypophyseal hormones at the age of 38 confirmed complete GH deficiency, whereas the other pituitary hormones were in the normal range (Table 1). She has now resumed exogenous GH therapy, and her weight is decreasing. The magnetic resonance of the pituitary region, not previously performed, was done with narrow scanning and gadolinium injection, showing a normal anatomy and size of the adenohypophysis for age and sex (−0.5 SDS) (22). A written informed consent was obtained from the proband's mother for herself and her daughter and from all the tested family members.

PCR amplification and sequencing of the GH1 gene

Genomic DNA was amplified by PCR using primers flanking the *GH1* gene and a proofreading *Taq* polymerase (Finnzymes, Espoo, Finland). The resulting 2.7-kb product was used as template for a series of nested PCRs using internal primers for the proximal promoter, the five exons, the four introns, and the untranslated regions of the *GH1* gene. PCR conditions and primer sequences are available upon request.

The resulting PCR products were visualized on a 2% agarose gel, purified from the gel using a Perfectprep Gel Cleanup system (Eppendorf, Hamburg, Germany) and used as template for sequencing in both senses with the Big-dye terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer.

The PCR product containing the IVS3 deletion in the heterozygous state was then cloned by the pMOS*Blue* T-vector kit (Amersham Biosciences, Little Chalfont, UK) following the manufacturer's instructions, and the two alleles were separately sequenced.

Synthesis and amplification of GH cDNA

Total RNA was extracted from lymphocytes by RNAwiz (Ambion, Austin, TX). cDNA was obtained by RT-PCR using the RETROscript Kit (Ambion) and amplified with three different couples of primers designed to amplify different parts of the wild-type cDNA (Fig. 1).

The products were extracted from a 2% agarose gel and directly sequenced.

Plasmid preparation and cell transfection

The 2700-bp PCR product encompassing the entire *GH1*, obtained from the proband's genomic DNA, was cloned into the pMOS plasmid (pMOSBlue T-vector kit; Amersham). Two clones containing the wild-type and the mutated allele, respectively, were selected by sequencing. A fragment of 2 kb containing the whole *GH1* sequence was released by digestion with *Bam*HI, purified from gel by the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany), and inserted into the pcDNA3.1(+) expression vector (Invitrogen, Chatsworth, CA) previously digested with *Bam*HI.

The *in vitro* mutagenized mutΔ7 was generated from the wild-type *GH1*-carrying plasmid by the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using two mismatch complementary oligonucleotides.

DH101F′ competent cells were then transformed with the different constructs and grown on LB/ampicillin media.

Plasmid DNA was extracted (Plasmid Midiprep; QIAGEN), sequenced, and used for transient transfection of the rat pituitary GH4C1 cell line (American Type Culture Collection, Rockville, MD). Cells were grown to approximately 80% confluence in 35-mm dishes and transfected with 2 μg of each construct using the Fugene 6 transfection

TABLE 1. Clinical and hormonal data of the two IGHD II patients

	Age (yr)	HSDS	BMI SDS	GH peak (ng/ml)	IGF-I (ng/ml)	IGF-I SDS (ng/ml)	Free T_4 (pg/ml)	TSH (μU/ml)	Cortisol (ng/ml)
Child									
h	0.16	−3.4	0.0						
h	0.42	−4.9	−0.7						
A	0.58	−5.8	−0.8	0.4[a]/0.4[b]	<15	−5.0	11.5	2.92	75.5
B	1.6	−2.9	−2.1	NA	38.9	−2.5	12.4	5.9	80.2
Mother									
h	2.0	−5.0							
A	4.6	−6.9	−0.4	3.0[a]/3.6[c]	NA	NA	NA	NA	105.4
B	5.6	−5.6	−1.2	NA	NA	NA	NA	NA	NA
C	16	−3.0	4.3	NA	NA	NA	14.5	4.2	NA
D	38	−2.7	6.7	0.5[d]	50	−4.0	15.4	1.5	148.2
E	39	−2.7	5.7	NA	164	−1.0	16.2	1.3	NA

NA, Not available; BMI SDS, body mass index SD score calculated from BMI Rolland-Cachera charts in pediatric age (19) and from BMI charts from American National Health and Nutrition Survey data in adulthood age (20); HSDS, height SD score; h, historical data; A, diagnosis; B, after 1 yr of GH treatment; C, stop of GH; D, restart of GH; E, after 1 yr of GH restart. Normal ranges: peak GH above 10 μg/ml; IGF-I 55–327 (0–1 yr), 51–303 (1–2 yr), 109–284 (36–40 yr); free T_4 11–20 (0–1 yr), 9–17 (1–6 yr), 9.3–17 (>18 yr); TSH 1.36–8.8 (0–1 yr), 0.85–6.5 (1–6 yr), 0.27–4.2 (>18 yr); cortisol 50–250 (early morning).

[a] Arginine stimulation test.

[b] Desametazone stimulation test.

[c] Insulin stimulation test.

[d] GHRH + arginine stimulation test.

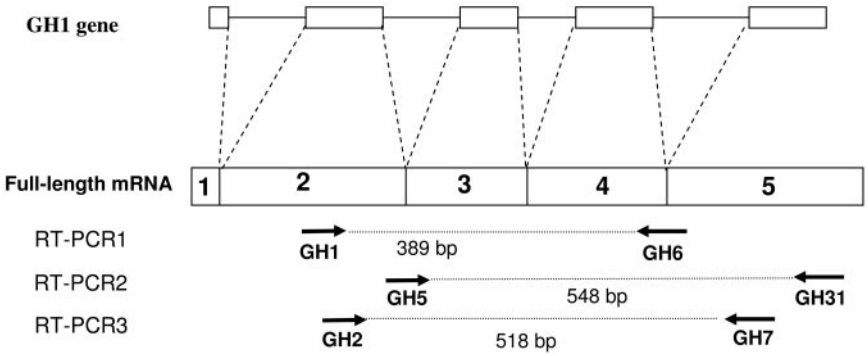

FIG. 1. Scheme of *GH1* pre-mRNA splicing showing the full-length mRNA and the RT-PCR products. RT-PCR1 performed with primers GH1 (5′-TTCCCAACCATTCCCTTATC-3′) and GH6 (5′-TCTTCCAGCCTCCCCATCAGC-3) amplifies part of exon 2, exon 3, and exon 4 allowing detection of exon 3 skipped products. RT-PCR2 performed with primers GH5 (5′-TACCAGGAGTTTGAAGAAGC-3′) and GH31 (5′-TCCAGGGCCAGGAGAGGCACTGGGG-3′) amplifies exon 3, exon 4, and exon 5 allowing detection of exon 4 skipped products. RT-PCR3 performed with primers GH2 (5′-CGTCTGCACCAGCTGGCCTTT-3′) and GH7 (5′-AAGCCACAGCTGCCCTCCACAGA-3′) amplifies part of exon 2, exon 3, exon 4, and part of exon 5 allowing detection of both exon 3 and exon 4 skipped products.

reagent (Roche), following the manufacturer's instructions. Total RNA was purified from rat cells 48 h after transfection by RNeasy Mini Kit (QIAGEN) and reverse transcribed.

Results

Sequencing of the GH1 gene

Sequencing of the *GH1* gene in the proband of an IGHD II family revealed the presence of a heterozygous 22-bp deletion in IVS3 (Fig. 2). The deletion is flanked by a stretch of five Cs (nt 53–57 of the IVS3) at the 5′ side and of three Cs (nt 75–77) at the 3′ side. Because three of these Cs are retained in the deleted allele, it is not possible to exactly define the limits of the deletion. We arbitrarily named the mutation as IVS3 del+56–77. The deletion includes the IVS3 BPS, which extends from nt 67 to nt 73 of IVS3 (between −25 and −20 from the 3′ splice site) (11). The same deletion was detected in the proband's mother (subject II-2, Fig. 3A), whereas it was absent in all the tested unaffected family members.

To identify the origin of the deletion, all the family members were typed for previously described promoter polymorphisms (23, 24), and *GH1* haplotype transmission was deduced from family segregation (Fig. 3A). The 22-bp deletion was carried by a *GH1* haplotype (−278T/−75A/−57G/−6G/+3G) that subject II-2 inherited from her father (I-1). The same haplotype was transmitted without the deletion from I-1 to II-3, II-5, and II-6. Thus, IVS3 del+56–77 is a *de novo* mutation in II-2.

Analysis of GH1 transcripts in lymphocytes

Ectopic transcript analysis was performed on cDNA from lymphocyte mRNA of all the family members. Two couples of primers were specifically designed to detect an aberrant splicing involving either exon 3 (RT-PCR1) or exon 4 (RT-PCR2), immediately 5′ and 3′ of the intron with the deletion (Fig. 1).

After amplification with RT-PCR1 primers (Fig. 3B), a band of 389 bp corresponding to the wild-type full-length cDNA was detected in all the individuals. In addition, the affected subjects III-1 and II-2 showed a smaller band of 269 bp with approximately the same intensity of the full-length band. This 269-bp band was scarcely detectable in the normal-height members and corresponded to the 17.5-kDa isoform lacking the entire exon 3. A faint 344-bp band, corresponding to the 20-kDa isoform, was visible in all the subjects.

Most of the previously reported BPS mutations in humans lead to either the partial or total skipping of the exon that follows the mutated intron (25–28) or to the retention of the intron carrying the mutated BPS (29). The presence of aberrant splicing products involving exon 4 was therefore investigated by RT-PCR2 (Fig. 3C). A unique band of 548 bp, corresponding to the full-length transcript, was detected both in the affected and unaffected family members.

Thus, the information deriving from lymphocyte mRNA

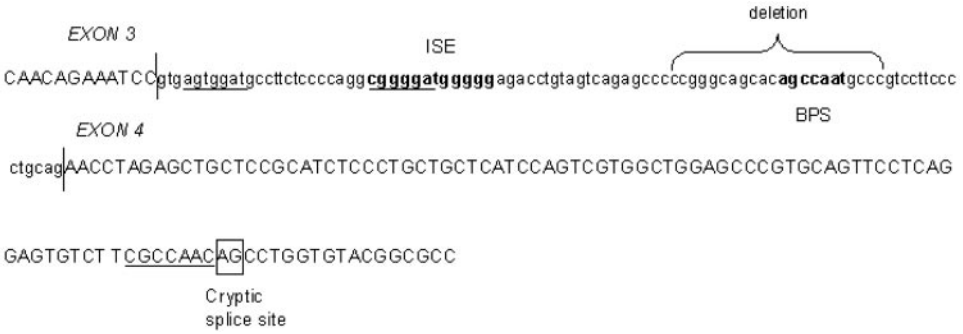

FIG. 2. Sequence of GH1 IVS3 and of part of exons 3 and 4. The intron splicing enhancer (ISE) and the BPS are in *bold*. The putative cryptic splice site is *boxed*. In IVS3 and exon 4, the cryptic BPSs are *underlined*.

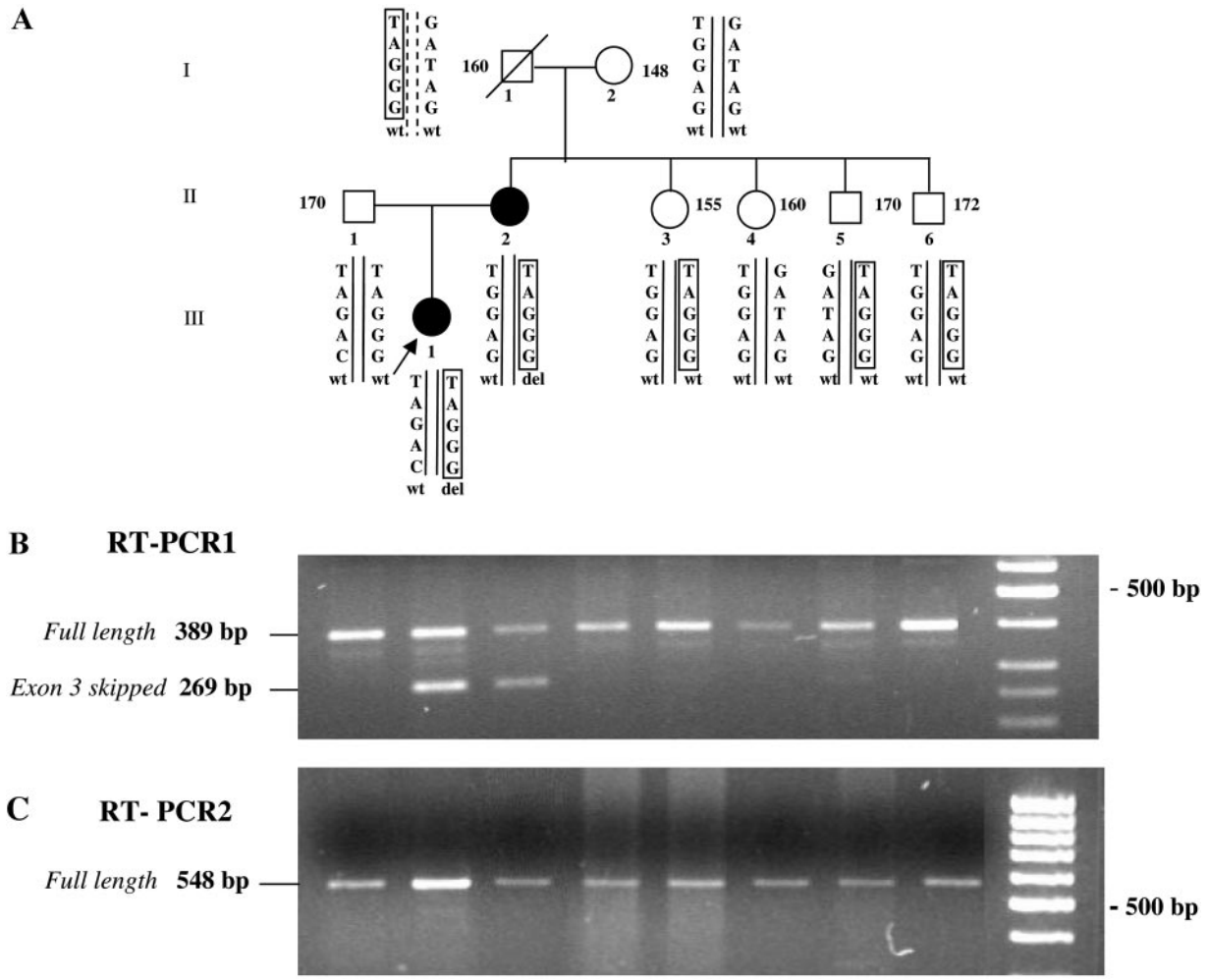

FIG. 3. A, Pedigree of the IGHD II family. *Black symbols* indicate affected individuals. For each member are indicated the stature (expressed in centimeters; only in unaffected individuals), the GH1 promoter haplotypes resulting, from top to bottom, from the allelic combinations of single-nucleotide polymorphisms −278G/T, −75A/G, −57T/G, −6A/G, +3G/C, and the IVS3 sequence (wt, wild-type; del, IVS3 del+56–77 mutation). From family segregation, it was possible to reconstruct both *GH1* haplotypes (indicated with a *broken line*) for the deceased individual I-1. The haplotype in which the *de novo* mutation occurred is *boxed*. The proband is indicated by an *arrow*. B, RT-PCR1 performed on lymphocyte mRNA; C, RT-PCR2 performed on lymphocyte mRNA. The gel lanes are positioned under the corresponding individual. All the visible bands were extracted from the gel and sequenced.

analysis was that the IVS3 deletion induces the production of a remarkable amount of mRNA lacking exon 3.

In vitro expression of the GH1 allele harboring the IVS3 del+56–77 mutation

To assess whether the skipping of exon 3 was the only aberrant product generated by the del+56–77 mutation, we analyzed the transcripts of the mutated allele in pituitary cells *in vitro*. To this purpose, we transfected GH4C1 rat pituitary cells with an expression vector containing either the normal *GH1* allele (wt-GH1) or the *GH1* allele with the IVS3 del+56–77 mutation (del-GH1). Because the two constructs were independently transfected, this analysis also allowed us to discriminate the products of the mutant and of the wild-type allele, which were coamplified in the lymphocyte cDNA.

The mRNAs from transfected cells were reverse transcribed and amplified with RT-PCR1 and RT-PCR2 primers. These are specific for the human *GH1* and did not amplify the rat mRNA from untransfected GH4C1 cells (Fig. 4, lane 2).

RT-PCR1 on the wt-GH1 mRNA yielded three bands corresponding to three *GH1* transcripts (Fig. 4A, lane 3) also detected in the lymphocytes of normal subjects, *i.e.* the main 22-kDa full-length transcript (389 bp), the 20-kDa isoform (344 bp), and the 17.5-kDa isoform (269 bp). On the contrary, the del-GH1 construct generated exclusively one intense band corresponding to the exon 3 skipped (17.5-kDa) transcript (Fig. 4A, lane 4).

When amplified with RT-PCR2 primers (Fig. 4B), which do not anneal to the exon 3 skipped product, the cDNA from cells transfected with wt-GH1 only contained the full-length transcript (lane 3), whereas del-GH1 generated three differently spliced mRNAs (lane 4) corresponding to 1) the full-length mRNA (548 bp), 2) a 462-bp transcript in which the first 86 bp of the exon 4 were deleted, and 3) a 383-bp transcript in which the 165 bp of exon 4 were absent. Thus, in

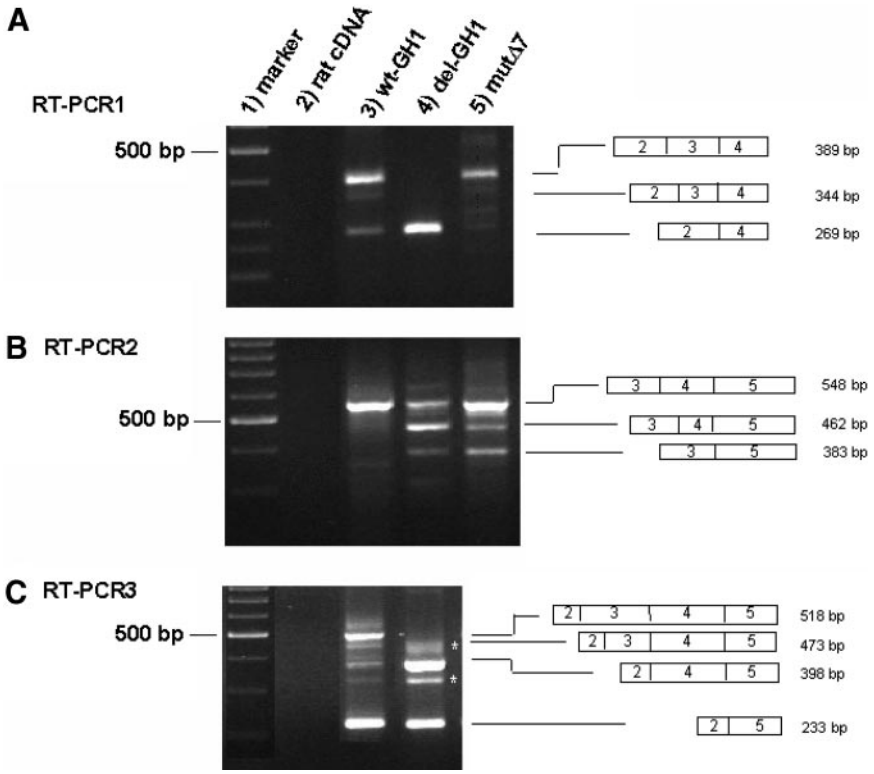

FIG. 4. RT-PCR performed on the mRNA extracted from GH4C1 pituitary cells: untransfected (rat cDNA; lane 2), transfected with the wild-type *GH1* gene (wt-GH1; lane 3), transfected with the *GH1* allele harboring the IVS3 del+56–77 mutation (del-GH1; lane 4), and transfected with the mutagenized *GH1* gene harboring the 7-bp BPS deletion (mutΔ7; lane 5). The RT-PCR was performed with the three couples of primers of RT-PCR1 (A), RT-PCR2 (B), and RT-PCR3 (C). Each PCR product was eluted from the gel and characterized by sequencing. The result is outlined on the *right* of each band. *, Heteroduplex molecules formed by mispairing of the 398- and 233-bp fragment.

pituitary cells transfected with the deleted allele, it was possible to detect two novel aberrantly spliced isoforms involving exon 4 that were not visible in the patients' lymphocytes and in pituitary cells transfected with the wild-type allele. Moreover, the RT-PCR2 primers showed that the mutant allele also produces a small amount of the full-length mRNA.

To investigate which of the above described alternatively spliced fragments was the direct consequence of the BPS deletion, the wt-GH1 plasmid was mutagenized to obtain a sequence lacking exclusively the 7 bp of the BPS consensus (mutΔ7). The cDNA produced by mutΔ7 showed only the aberrant exon 4 skipped fragments as expected for a BPS mutation (Fig. 4, A and B). As observed for del-GH1, the mutΔ7 construct also produced a visible amount of the full-length cDNA.

A third set of primers (RT-PCR3) located within exons 1–2 and exon 5 was designed to amplify all the isoforms in the same reaction (Fig. 1). Because we did not perform a quantitative PCR, we can only roughly estimate the relative amount of the different isoforms, with the limitation that smaller fragments are in any case preferentially amplified. Four fragments were visible on a 2% agarose gel in correspondence of the deleted allele (Fig. 4C, lane 4). The two bands of higher intensity corresponded to the 17.5-kDa isoform (398 bp) and to a smaller transcript lacking both exons 3 and 4 (233 bp), encoding the normal 11.3-kDa isoform (2). The latter is detectable only under the RT-PCR3 conditions (Fig. 1) and was visible with a similar intensity also among the wt-GH1 transcripts (Fig. 4C, lane 3). The two fainter bands corresponded to heteroduplex molecules formed by mispairing of the 398- and 233-bp fragments. The full-length cDNA and the transcripts with aberrant exon 4 skipping were not visible in this PCR. The wt allele (Fig. 4C, lane 3) yielded, besides the 233-bp fragment, a strong band corresponding to the full-length cDNA and two fainter bands corresponding to the 17.5- and 20-kDa isoforms.

Discussion

Mutations in the *GH1* splicing elements that result in an increased level of exon 3 skipped transcripts encoding the 17.5-kDa isoform are a common cause of autosomal dominant type II IGHD and severe growth failure (4–12).

Here we report a new mutation, consisting of a 22-bp deletion in the IVS3 (IVS3 del+56–77) causing IGHD II in a child and her mother. The deletion completely removes the reported BPS (AGCCAAT) (11) located between −20 and −25 upstream of the intron 3/exon 4 junction (Fig. 2) matching five of the seven residues of the weakly defined mammalian BPS ($Y_{81}NY_{100}T_{87}R_{81}A_{100}Y_{94,}$ where Y represents pyrimidine, R represents purine, and N represents any base) (30). The A at position −21 (underlined) likely represents the highly conserved adenine involved in the lariat formation with the donor splice site.

Lymphocyte mRNA analysis showed that this mutation induced a splicing pattern analogous to that observed in most cases of IGHD II; the proband and her mother showed two principal mRNA species approximately in equal amount, namely the full-length mRNA (encoded by the normal allele) and an aberrant splicing product with the skipping of exon 3 (encoded by the mutant allele) (Fig. 3B).

Accordingly, the clinical phenotype determined by the 22-bp IVS3 deletion correlated with that observed in other IGHD II patients harboring splice site mutations. The phe-

notype of IGHD II patients is heterogeneous. On average, patients carrying splice site mutations show earlier age of onset of the growth failure that is progressive and more severe than patients carrying missense mutations (8, 31, 32). However, phenotype variability is present also among affected members of the same family (8, 31, 33) showing that the GH-dependent growth during childhood is individually different and modified by several factors, most of them still unknown. Both our patients showed an early postnatal onset of the growth failure that was progressive and severe. Patient III-1 showed an arrest of growth at the age of 4 months and was diagnosed at 7 months (−5.8 SDS); her mother was diagnosed at 4.6 yr (−6.9 SDS) and showed a height of −5 SDS at the age of 2 yr (no information is available at an earlier age). The clinical phenotype was somewhat less severe in the mother; GH secretion after provocative test, completely absent in the daughter, was severely but not completely reduced in the mother at the childhood age diagnosis, and the size of the pituitary gland was normal in the mother at the adult age, whereas it was at the lower limit size in the child. When retested in adulthood, the mother had no GH response to the strong GHRH plus arginine stimulation test, suggesting an exhausted function of the somatotropic cells along the years. Conversely, she did not develop other pituitary hormone deficiencies as described in some adult IGHD II patients (32).

The mutation described here is different from other intronic mutations detected in IGHD II families (4–12) because it leaves intact the splice junctions and the enhancers, whereas it completely removes the BPS. On the basis of what has been observed in other human diseases (25–29), we predicted that a mutation involving the BPS would induce the total or partial skipping of exon 4. However, lymphocyte mRNA analysis as well as analysis on the mRNA extracted from rat pituitary cells transfected with del-GH1 revealed that the major transcript was the exon 3 skipped. Two aberrantly spliced isoforms expected for a BPS mutation were detectable exclusively in experimental conditions where the exon 3 skipped mRNA was excluded from the possible targets (Fig. 4B, lane 4). The first isoform, lacking the entire exon 4, encodes for a putative mature peptide depleted of amino acids 72–126. The other was an mRNA devoid of the first 86 bases of exon 4, and it causes a frameshift leading to a putative protein differing from the wild type from amino acid 72 and prematurely truncated at residue 77. It likely results from the use of a cryptic acceptor splice site at position 85–86 of exon 4. The recognition of this splice site might be mediated by the presence of a putative cryptic BPS (CGCCAAC), matching six of the seven bases of the consensus sequence, located between nt 78 and 84 of exon 4 (Fig. 2).

The content of the abnormal mRNA lacking exon 4, or a part of it, is likely very low in the patients' lymphocytes compared with the normal-sized and to the exon 3 skipped mRNA and can therefore be visualized only under specific experimental conditions. One of the reasons for the low content, at least for the prematurely truncated isoform, might be its rapid degradation owing to nonsense-mediated mRNA decay (34).

The two aberrant splicing events involving exon 4 are the direct consequence of the missing BPS, as demonstrated by the mutagenized construct lacking exclusively the 7 bp of the BPS consensus (Fig. 4B, lane 5). Conversely, the high content of the exon 3 skipped isoform remains to be explained. In a recent report, Ryther and colleagues (35) demonstrated that the overall size of IVS3 is crucial for exon 3 inclusion in the mRNA and showed that deletions of 12–14 bp are sufficient to increase exon 3 skipping. They suggested that this is because the decreased IVS3 size rather than the deletion of specific sequences. We can thus speculate that the main effect of the 22-bp deletion, *i.e.* the increased exon 3 skipping with production of the IGHD II-specific pathological amount of the 17.5-kDa isoform, is the consequence of the remarkably decreased size of IVS3 that overwhelms the effect caused by the BPS deletion.

However, in the deletion mutants reported by Ryther *et al.* (35), the IVS3 BPS was unaltered and mediated the lariat formation with the IVS2 donor splice site. In our case, it is not clear how the two mutants lacking the IVS3 branch site (namely the del+56–77 allele and the mutagenized mutΔ7) can correctly process the IVS3 splicing necessary for the exon 3 skipped and the full-length mRNA (36). A similar situation was reported for other disease-causing BPS mutations where the mutated allele also produced the wild-type isoform in addition to the isoform with an aberrant splicing (29, 37, 38). An obvious explanation for these observations is the use of an alternative BPS elsewhere in the IVS3 efficiently replacing the deleted one. Two sequences located between −40/−46 and −61/−67 from the GH1-IVS3 acceptor splice site in the deleted allele, within a distance compatible with a BPS function (39), match the consensus more than others. However, the del-GH1 plasmid mutagenized at the highly conserved A of either or both of these two cryptic BPSs yielded the same splicing pattern (data not shown) as the nonmutagenized construct (Fig. 4). It is thus conceivable that other sequences with less homology to the consensus might act as a cryptic BPS. In fact, although several reported BPS mutations cause severe phenotypes, it has also been demonstrated that a mutated BPS can be replaced by a cryptic BPS with no resemblance to the consensus branch site, apart from the A residue necessary for the lariat formation (40).

In conclusion, despite the absence of the canonical BPS, the exon 3 skipped is the principal transcript and the cause of IGHD II in our patients. Thus, the context in which the BPS mutation is located, *i.e.* the 22-bp deletion, has a greater phenotypic influence than the BPS mutation itself. It is unlikely that the two isoforms with an aberrant splicing of exon 4 also contribute to the IGHD II phenotype because they are presumably poorly expressed.

Acknowledgments

We are grateful to the patients and their relatives. We thank Dr. T. Vago and Dr. G. Baldi from the Endocrine Laboratory of Luigi Sacco Hospital for hormonal dosage and Dr. M. Bevilacqua from the Endocrinology Department of Luigi Sacco Hospital for cooperation on adult patient management and scientific support.

Received July 29, 2005. Accepted December 13, 2005.

Address all correspondence and requests for reprints to: Mara Giordano, Ph.D., Dipartimento di Scienze Mediche, Via Solaroli 17, 28100 Novara, Italy. E-mail: giordano@med.unipmn.it.

This work was supported by grants from Pfizer, Italian Network for Molecular Biology in Pediatric Endocrinology, Eastern Piedmont University, the Italian Ministry for University and Research (Cofin 2003), and Cariplo Foundation. M.G. and D.V. are Ph.D. fellows of Dottorato in Medicina Molecolare, Eastern Piedmont University.

References

1. **Stewart TA, Clift S, Pitts-Meek S, Martin L, Terrell TG, Liggitt D, Oakley H** 1992 An evaluation of the functions of the 22-kilodalton (kDa), the 20-kDa, and the N-terminal polypeptide forms of human growth hormone using transgenic mice. Endocrinology 130:405–414
2. **Procter AM, Phillips 3rd JA, Cooper DN** 1998 The molecular genetics of growth hormone deficiency. Hum Genet 103:255–272
3. **Binder G** 2002 Isolated growth hormone deficiency and the GH-1 gene: update 2002. Horm Res 58(Suppl 3):2–6
4. **Binder G, Ranke MB** 1995 Screening for growth hormone (GH) gene splice-site mutations in sporadic cases with severe isolated GH deficiency using ectopic transcript analysis. J Clin Endocrinol Metab 80:1247–1252
5. **Cogan JD, Ramel B, Lehto M, Phillips 3rd J, Prince M, Blizzard RM, de Ravel TJ, Brammert M, Groop L** 1995 A recurring dominant negative mutation causes autosomal dominant growth hormone deficiency: a clinical research center study. J Clin Endocrinol Metab 80:3591–3595
6. **Missarelli C, Herrera L, Mericq V, Carvallo P** 1997 Two different splice site mutations in the growth hormone gene causing autosomal dominant growth hormone deficiency. Hum Genet 101:113–117
7. **Hayashi Y, Kamijo T, Yamamoto M, Ohmori S, Phillips 3rd JA, Ogawa M, Igarashi Y, Seo H** 1999 A novel mutation at the donor splice site of intron 3 of the GH-1 gene in a patient with isolated growth hormone deficiency. Growth Horm IGF Res 9:434–437
8. **Binder G, Keller E, Mix M, Massa GG, Stokvis-Brantsma WH, Wit JM, Ranke MB** 2001 Isolated GH deficiency with dominant inheritance: new mutations, new insights. J Clin Endocrinol Metab 86:3877–3881
9. **Takahashi I, Takahashi T, Komatsu M, Sato T, Takada G** 2002 An exonic mutation of the GH-1 gene causing familial isolated growth hormone deficiency type II. Clin Genet 61:222–225
10. **Moseley CT, Mullis PE, Prince MA, Phillips 3rd JA** 2002 An exon splice enhancer mutation causes autosomal dominant GH deficiency. J Clin Endocrinol Metab 87:847–852
11. **Cogan JD, Prince MA, Lekhakula S, Bundey S, Futrakul A, McCarthy EM, Phillips 3rd JA** 1997 A novel mechanism of aberrant pre-mRNA splicing in humans. Hum Mol Genet 6:909–912
12. **McCarthy EM, Phillips 3rd JA** 1998 Characterization of an intron splice enhancer that regulates alternative splicing of human GH pre-mRNA. Hum Mol Genet 7:1491–1496
13. **De Vos A, Ultsch M, Kossiakoff A** 1992 Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255:306–312
14. **Graves TK, Patel S, Dannies PS, Hinkle PM** 2001 Misfolded growth hormone causes fragmentation of the Golgi apparatus and disrupts endoplasmic reticulum-to-Golgi traffic. J Cell Sci 114:3685–3694
15. **Lee MS, Wajnrajch MP, Kim SS, Plotnick LP, Wang J, Gertner JM, Leibel RL, Dannies PS** 2000 Autosomal dominant growth hormone (GH) deficiency type II: the Del32–71-GH deletion mutant suppresses secretion of wild-type GH. Endocrinology 141:883–890
16. **McGuinness L, Magoulas C, Sesay AK, Mathers K, Carmignac D, Mannerville JB, Christian H, Phillips 3rd JA, Robinson IC** 2003 Autosomal dominant growth hormone deficiency disrupts secretory vesicles *in vitro* and *in vivo* in transgenic mice. Endocrinology 144:720–731
17. **Duquesnoy P, Simon D, Netchine I, Dastot F, Sobrier ML, Goossens M, Cernichow P, Amselem S** 1998 Familial isolated growth hormone deficiency with slight height reduction due to a heterozygote mutation in GH gene. Program of the 80th Annual Meeting of The Endocrine Society, New Orleans, LA, 1998, p 295 (Abstract P2-202)
18. **Deladoey J, Stocker P, Mullis PE** 2001 Autosomal dominant GH deficiency due to an Arg183His GH-1 gene mutation: clinical and molecular evidence of impaired regulated GH secretion. J Clin Endocrinol Metab 86:3941–3947
19. **Rolland-Cachera MF, Sempe M, Guillond-Bataille M** 1984 Adiposity indices in children. Am J Clin Nutr 36:178–184
20. **Simopoulos AP, Van Itallie TB** 1984 Body weight, health and longevity. Ann Intern Med 100:285–295
21. **Argyropoulous M, Perignon F, Brauner R, Brunelle F** 1992 Magnetic resonance imaging in the diagnosis of growth hormone deficiency. J Pediatr 120: 886–891
22. **Tsunoda A, Okuda O, Sato K** 1997 MR height of the pituitary gland as a function of age and sex: especially physiological hypertrophy in adolescence and in climacterium. AJNR Am J Neuroradiol 18:551–554
23. **Giordano M, Marchetti C, Chiorboli E, Bona G, Momigliano-Richiardi P** 1997 Evidence for gene conversion in the generation of extensive polymorphism in the promoter of the growth hormone gene. Hum Genet 100:249–255
24. **Wagner JK, Ebl A, Cogan J D, Prince MA, Phillips III JA, Mullis PE** 1997 Allelic variations in the human growth hormone-1 gene promoter of growth hormone-deficient patients and normal controls. Eur J Endocrinol 137:474–481
25. **Maslen C, Babcock D, Raghunath M, Steinmann B** 1997 A rare branch-point mutation is associated with missplicing of fibrillin-2 in a large family with congenital contractural arachnodactyly. Am J Hum Genet 60:1389–1398
26. **Hamlington JD, Clough MV, Dunston JA, McIntosh I** 2000 Deletion of a branch-point consensus sequence in the LMX1B gene causes exon skipping in a family with nail patella syndrome. Eur J Hum Genet 8:311–314
27. **Di Leo E, Panico F, Tarugi P, Battisti C, Federico A, Calandra S** 2004 A point mutation in the lariat branch point of intron 6 of NPC1 as the cause of abnormal pre-mRNA splicing in Niemann-Pick type C disease. Hum Mutat 24:440
28. **Khan SG, Metin A, Gozukara E, Inui H, Shahlavi T, Muniz-Medina V, Baker CC, Ueda T, Aiken JR, Schneider TD, Kraemer KH** 2004 Two essential splice lariat branchpoint sequences in one intron in a xeroderma pigmentosum DNA repair gene: mutations result in reduced XPC mRNA levels that correlate with cancer risk. Hum Mol Genet 13:343–352
29. **Kuivenhoven JA, Weibusch H, Pritchard PH, Funke H, Benne R, Assmann G, Kastelein JJ** 1996 An intronic mutation in a lariat branchpoint sequence is a direct cause of an inherited human disorder (fish-eye disease). J Clin Invest 98:358–364
30. **Smith CW, Porro EB, Patton JG, Nadal-Ginard B** 1989 Scanning from an independently specified branch point defines the 3′ splice site of mammalian introns. Nature 342:243–247
31. **Fofanova OV, Evgrafov OV, Polyakov AV, Poltaraus AB, Peterkova VA, Dedov II** 2003 A novel IVS2 -2A>T splicing mutation in the *GH-1* gene in familial isolated growth hormone deficiency type II in the spectrum of other splicing mutations in the Russian population. J Clin Endocrinol Metab 88: 820–826
32. **Mullis PE, Robinson IC, Salemi S, Eble A, Besson A, Vuissoz JM, Deladoey J, Simon D, Czernichow P, Binder G** 2005 Isolated autosomal dominant growth hormone deficiency: an evolving pituitary deficit? A multicenter follow-up study. J Clin Endocrinol Metab 90:2089–2096
33. **Kamijo T, Hayashi Y, Shimatsu A, Kinoshita E, Yoshimoto M, Ogawa M, Seo H** 1999 Mutations in intron 3 of GH-1 gene associated with isolated GH deficiency type II in three Japanese families. Clin Endocrinol 51:355–360
34. **Alonso CR** 2005 Nonsense-mediated RNA decay: a molecular system micromanaging individual gene activities and suppressing genomic noise. Bioessays 27:463–466
35. **Ryther RC, Flynt AS, Harris BD, Phillips 3rd JA, Patton JG** 2004 GH1 splicing is regulated by multiple enhancers whose mutation produces a dominant-negative GH isoform that can be degraded by allele-specific siRNA. Endocrinology 145:2988–2996
36. **Black DL** 2003 Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 72:291–336
37. **Burrows NP, Nicholls AC, Richards AJ, Luccarini C, Harrison JB, Yates JR, Pope FM** 1998 A point mutation in an intronic branch site results in aberrant splicing of COL5A1 and in Ehlers-Danlos syndrome type II in two British families. Am J Hum Genet 63:390–398
38. **Zhu X, Chung I, O'Gorman MR, Scholl PR** 2001 Coexpression of normal and mutated CD40 ligand with deletion of a putative RNA lariat branchpoint sequence in X-linked hyper-IgM syndrome. Clin Immunol 99:334–339
39. **Helfman DM, Ricci WM** 1989 Branch point selection in alternative splicing of tropomyosin pre-mRNAs. Nucleic Acid Res 17:5633–5650
40. **Padgett RA, Konarska MM, Aebi M, Hornig H, Weissmann C, Sharp PA** 1985 Nonconsensus branch-site sequences in the *in vitro* splicing of transcripts of mutant rabbit β-globin genes. Proc Natl Acad Sci USA 82:8349–8353

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.