Novel Mutations in the GH Gene (*GH1*) Uncover Putative Splicing Regulatory Elements

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Mutations affecting exon 3 splicing are the main cause of autosomal dominant Isolated GH Deficiency II (IGHDII) by increasing the level of exon 3-skipped mRNA encoding the functionally inactive dominant-negative 17.5-kDa isoform. The exons and introns of the gene encoding GH (*GH1*) were screened for the presence of mutations in 103 sporadic isolated GH deficiency cases. Four different variations within exon 3 were identified in 3 patients. One carried c.261C>T (p.Pro87Pro) and c.272A>T (p.Glu91Val), the second c.255G>A (p.Pro85Pro) and c.261 C>T, and the third c.246G>C (p.Glu82Asp). All the variants were likely generated by gene conversion from an homologous gene in the *GH1* cluster. In silico analysis predicted that positions c.255 and c.272 were included within 2 putative novel exon splicing enhancers (ESEs). Their effect on splicing was confirmed in vitro. Constructs bearing these 2 variants induced consistently higher levels both of transcript and protein corresponding to the 17.5-kDa isoform. When c.255 and c.272 were combined in *cis* with the c.261 variant, as in our patients, their effect was weaker. In conclusion, we identified 2 variations, c.255G>A and c.272A>T, located in 2 novel putative exon splicing enhancers and affecting *GH1* splicing in vitro by increasing the production of alternatively spliced isoforms. The amount of aberrant isoforms is further regulated by the presence in *cis* of the c.261 variant. Thus, our results evidenced novel putative splicing regulatory elements within exon 3, confirming the crucial role of this exon in mRNA processing. **(*Endocrinology* 155: 1786–1792, 2014)**

The *GH1* gene is located on chromosome 17q23 within a cluster of 5 highly homologous genes, all consisting of 5 exons and 4 introns, including the placentally expressed *GH2*, 2 chorionic somatomammotropin genes *CSH1* and *CSH2*, and a pseudogene *CSHP1* (1). When correctly spliced, *GH1* produces the 22-kDa isoform that includes all the 5 exons with the complete biological activity of GH (2, 3). Despite the correct processing, even under normal conditions, a small percentage of alternatively spliced isoforms are produced. The presence of an in-frame cryptic splice site within exon 3 gives rise to a transcript lacking the first 45 bp of exon 3 and encodes a shorter active isoform of 20 kDa, representing 5%–10% of GH transcripts (4). A 17.5-kDa isoform (representing 0.1%–5% of GH transcripts) is produced by the complete skipping of exon 3, thus lacking the entire loop connecting helix 1 and helix 2 in the tertiary structure of GH and generating a GH isoform with no biological activity (5). Trace amounts of the severely truncated isoforms of 11.3 and 7.4 kDa, which are biologically inactive, have also been identified being generated by the skipping of exons 3 and 4 or 2 to 4, respectively (6). Multiple mechanisms have evolved to maintain the small amounts of these aberrantly spliced isoforms, especially that encoding the 17.5-kDa protein. Because *GH1* has weak canonical splice sites, multiple *cis*-acting splicing regulatory elements (splicing

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Abbreviations: ASF/SF2, alternative splicing factor 1/pre-mRNA splicing factor SF2; ESE, exon splicing enhancer; IGHD, isolated GH deficiency; SR proteins, serine/arginine-rich proteins; SRp40, serine/arginine-rich splicing factor 5; TW2, Tanner-Whitehouse 2nd Edition.

enhancers) are essential to maintain the correct exon 3 definition through the activation of the canonical intron 2 and 3 splice sites and silencing of the cryptic sites. Two exon splicing enhancers (ESEs), ESE1, encompassing the first 7 bases of exon 3 (from c.172 to c.178), and ESE2 (from c.190 to c.204), located 12 nt upstream of the cryptic splice site in exon 3 (Figure 1A), and an intron splicing enhancer (ISE) within intron 3, have been well characterized (7–11).

Several mutations leading to aberrant splicing have been reported in isolated GH deficiency (IGHD) patients within these enhancer motifs (7, 8, 10, 11). The increased amount of the 17.5-kDa isoform exhibits a dominant negative effect both in tissue culture and transgenic mice experiments by disrupting the secretory pathway and trafficking of normal GH and other hormones, including ACTH (12, 13).

We here report the identification of variations within *GH1* exon 3 in sporadic IGHD patients and absent in a group of 205 normal stature controls and in the public databases. In silico analysis suggested that 2 of these variations affect exon 3 splicing, because they are located within putative ESEs. In vitro mRNA and Western blot analysis confirmed the deleterious effect of the single variations on splicing, suggesting the presence of further splicing regulatory elements within *GH1* exon 3.

Subjects and Methods

Subjects

A total of 103 sporadic patients with IGHD and 205 normal stature individuals, all belonging to the Italian population, were included in the genetic analysis. The subjects were referred to the clinical centers because they had a height less than or equal to −2 SDS or a height less than or equal to −1.8 SDS in combination with a height velocity over 1 year less than −1.5 SDS using the criteria of Tanner-Whitehouse (14). Patients with a known postnatal cause of acquired hypopituitarism were excluded. Skeletal maturation was estimated as bone age (radius, ulna, and short bone) with the TW2 (Tanner-Whitehouse 2nd Edition) method by a pediatric endocrinologist (15). They were all evaluated for GH serum levels after 2 provocative tests (with arginine or clonidine or insulin or glucagon or with GHRH + arginine (16). Traditionally, a diagnosis of GHD is supported by GH peaks less than 10 ng/mL both after the 2 different stimuli (17), or less than 20 ng/mL after the double provocative test with GHRH + arginine. The GHD patients fulfill these criteria and had a mean (±SD) secretion peak of 4.4 ± 2.5 ng/mL after the classical stimuli (n = 78) or 9.4 ± 5.8 ng/mL after the test with GHRH + arginine (n = 25). None of the GHD patients was deficient for

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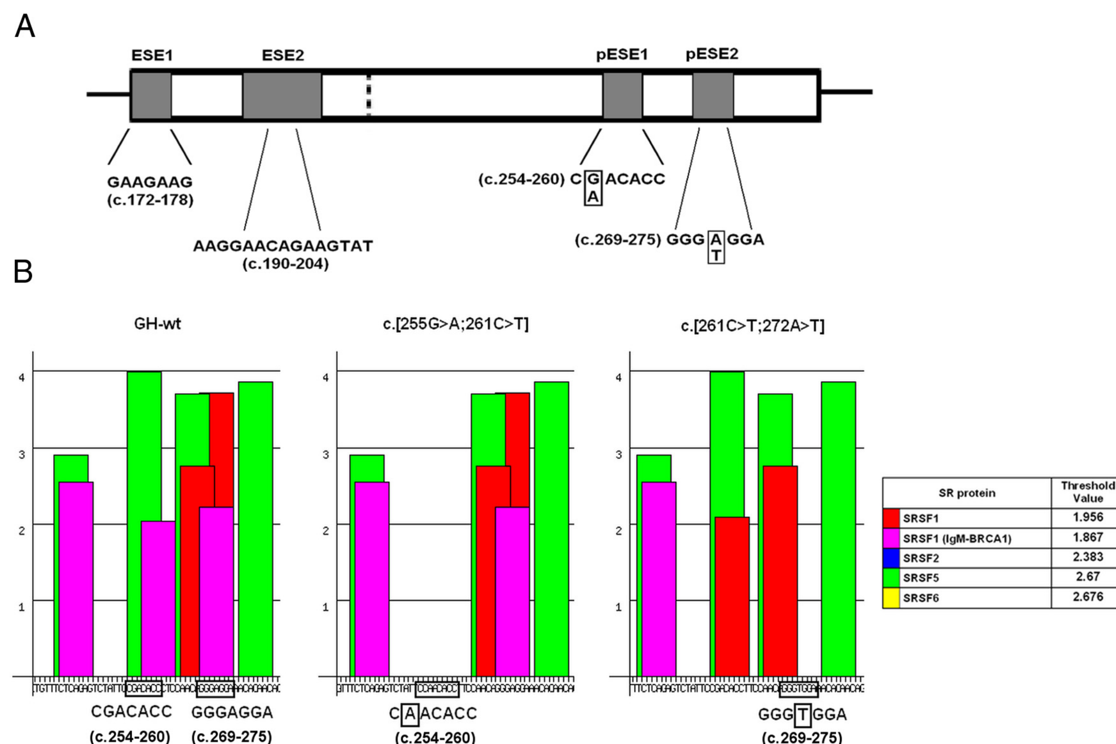

Figure 1. A, ESEs in exon 3. The 2 known ESEs (ESE1 and ESE2) are indicated. The cryptic splice site is at c.216 and is indicated with a dotted line. The newly identified putative ESEs are indicated as pESE1 and pESE2. The mutations identified in patient 1 and patient 2 in the pESEs are boxed. B, ESE finder analysis performed on the wild-type exon 3 and on the different exon 3 alleles identified in patients. The analysis using wild-type sequence revealed all the putative exon splicing enhancer sites within exon 3. ESE motifs with scores above the threshold for each SR protein are indicated in differently colored bars. The bar heights reflect the score of the motifs. Threshold values and color code for each of the different motifs are indicated in Table 1. The introduction of c.255G>A and c.272A>T variations caused the abolition of the 2 putative ESE elements at positions c.254–260 and c.269–275, respectively.

other pituitary hormones, and none had a documented family history of the disease or consanguineous parents. All the patients have been also screened for mutations in *GHRHR* (data not shown). Patients carrying mutations in this gene were not included in the IGHD cohort described here. Normal stature controls included University and Hospital staff, as well as medical students not tested for GH secretion levels. A written informed consent was obtained from the patient's parents, because they were all aged less than 18, and from the normal stature controls.

Detailed description of patients carrying the *GH1* variations

The variants identified in the three patients are reported in Table 1.

Case 1

This patient is a boy born at term with no perinatal complications. He came to our attention when he was 7.3 years old with a height of −2.7 SDS. He has normal stature parents: the father is −1.7 SDS and the mother −0.6 SDS. He was prepubertal. GHD was diagnosed based on low response to insulin tolerance test and clonidine provocative tests (8.9 and 8.6 ng/mL, respectively). The IGF-I level was 130 ng/mL. The bone age was delayed by 3.8 years. GH therapy was initiated with a good clinical response reaching a height of −1.7 SDS after 1.3 after 6 years.

Case 2

This case is a male born from nonconsanguineous parents by vaginal delivery after 41 weeks of gestation. At birth, he was adequate for gestational age (birth weight, 3330 g; length, 52 cm). Both parents presented normal height: father, 174 cm (−0.2 SDS) and mother, 155 cm (−1.3 SDS). He came to our attention at age 13 presenting short stature with a height of −3.4 SDS. His growth rate in the year preceding the diagnosis was 5.0 cm/y (−1.5 SDS). At the diagnosis, the pubertal stage was: pubic hair development 2, genital development 2, and bilateral testicular volume 5. The bone age was delayed (11.6, TW2 method). GH secretion peaks after stimulus with arginine and clonidine were 3.9 and 8.3 ng/mL, respectively, with an IGF-I level of 65 ng/mL. He promptly started the recombinant human GH replacement therapy (0.22 mg/kg·wk) and reached the height of −1.8 SDS after 4 years.

Case 3

This boy came for the first time to our attention for growth failure at the age of 16.5 years. At diagnosis, his stature was −2.7 SDS. He was born to nonconsanguineous parents after a normal pregnancy at 40 weeks of gestation, adequate for gestational age (birth weight, 2950 g). His father and mother presented normal (−0.5 and −0.3 SDS, respectively). The pubertal stage was: pubic hair development 3, genital development 3, and bilateral testicular volume 8 mL. The growth velocity was 4.5 cm/y (1.6 SDS, 1.6 SDS, but low when corrected for pubertal stage), and x-ray revealed a bone age (TW2) of 14.2 years. Laboratory analysis revealed a normal IGF-I level (180 ng/mL) and low level of circulating GH after 2 provocative tests (7.7 ng/mL with arginine and 3.7 with clonididne). The recombinant human GH replacement therapy (0.24 mg/kg·wk) was begun with a good response, because he reached the height of −0.7 SDS after 3 years.

Table 1. Exon 3 Variations Detected in the IGHD Patients

Patient	Variation Nucleotide	Amino acid	Within a predicted ESE
1	c.261C>T	(p.Pro87Pro)	No
	c.272A>T	(p.Glu91Val)	Yes
2	c.255G>A	(p.Pro85Pro)	Yes
	c.261C>T	(p.Pro87Pro)	No
3	c.246G>C	(p.Glu82Asp)	No

Predicted ESE by the software ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

Screening of *GH1*

Genomic DNA was amplified by PCR using previously described primers (18) and a proofreading Taq polymerase (Finnzymes). The resulting 2.7-kb product, including the whole *GH1*, was used as template for a series of nested PCRs using internal primers for the proximal promoter, 5 exons, 4 introns, and the untranslated regions of the *GH1*. These primers are designed specifically for the *GH1* and do not amplify other genes in the cluster. PCR conditions and primer sequences are available upon request. The PCR products were visualized on a 2% agarose gel and purified using ExoSAP-IT enzymatic PCR clean up system (Affymetrix). The purified products were then sequenced with the Big Dye Terminator kit (Applied Biosystems) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The PCR products containing the variations in the heterozygous state were then cloned into the plasmid vector pTZ57R/T using the InsTAclone PCR cloning kit (Fermentas), and the 2 alleles were separately sequenced.

Site-directed mutagenesis

The 2.7-kb fragment containing *GH1* was inserted into the pcDNA 3.1(+) expression vector (Invitrogen). The constructs bearing the single variants, namely 246C, 255A, 261T, and 272T, and the variants combined as in the patients, namely 261T/272T (patient 1) and 255A/261T (patient 2), were generated from the GH(wt)-pcDNA 3.1 plasmid by the QuikChange Site-Directed Mutagenesis kit from Stratagene using mismatch complementary primers containing the desired mutations. PCR conditions and primer sequences are available upon request. DH5a competent cells were transformed with the different constructs and grown on Luria Broth/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using Maxiprep kit (QIAGEN). The desired mutation was confirmed by sequencing.

Cell culture, transfection, and isolation of RNA

The GH4C1 rat pituitary cell line was used for the transfection experiments. The stock culture was grown in Ham's F10 medium (Gibco-Life Technologies) supplemented with 15% horse serum, 2.5% fetal bovine serum, 100-U/mL penicillin, and 100-μg/mL streptomycin in a 5% CO_2. A day before transfection, 4×10^5 cells were seeded into each well of a 6-well tissue culture plate in 2.5-mL medium. The wells were previously treated with 1:10 diluted poly-L-lysine solution (Sigma-Aldrich) to allow the cells to completely attach to the plate. At 50%–70% confluency, cells were transfected with 2.5-μg DNA of the

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wt-GH or the mutated constructs using the Trans IT-LT1 transfection reagent (Mirus Bio LLC). A green fluorescent protein control was used to test transfection efficiency. Forty-eight hours after transfection, total RNA was isolated and purified from the cells using the QIAGEN RNA mini kit (QIAGEN).

cDNA synthesis

cDNA was synthesized from 1.5 μg of RNA by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. The different transcripts produced by alternative splicing were analyzed using primers specific for *GH1* cDNA (Figure 2A). The RT-PCR was performed with primers GH2 (5′-CGTCTGCACCAGCTGGCCTTT-3′) and GH7 (5′-AAGCCACAGCTGCCCTCCACAGA-3′), which amplify part of exon 2, exon 3, exon 4, and part of exon 5, allowing detection of both exon 3- and exon 4-skipped products.

Western immunoblot analysis

CHO cells were transiently transfected with wt-GH and mutated constructs, as described above. After 48 hours, whole-cell lysates (WCL) were collected using the standard radio-immuno precipitation assay (RIPA) lysis buffer containing 0.1% sodium dodecyl sulfate (SDS). A total of 20 μg of WCL were separated on 15% SDS-PAGE gel and blotted on Immun-Blot PVDF membrane (Bio-Rad). Membranes were probed with a polyclonal rabbit antihuman GH antibody (Abnova) and detected with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Millipore). A polyclonal antiactin antibody (Sigma-Aldrich) was used to normalize the protein loading. Protein bands were visualized using enhanced chemiluminescence reagent (Thermo Scientific) with image capture performed using a charge-coupled device camera linked to ChemiDoc apparatus (Bio-Rad).

CHO cells were used instead of GH4C1 cells for protein analysis, because the GH4C1 showed many unspecific bands after Western blotting, likely due to cross-reactions with endogenous proteins.

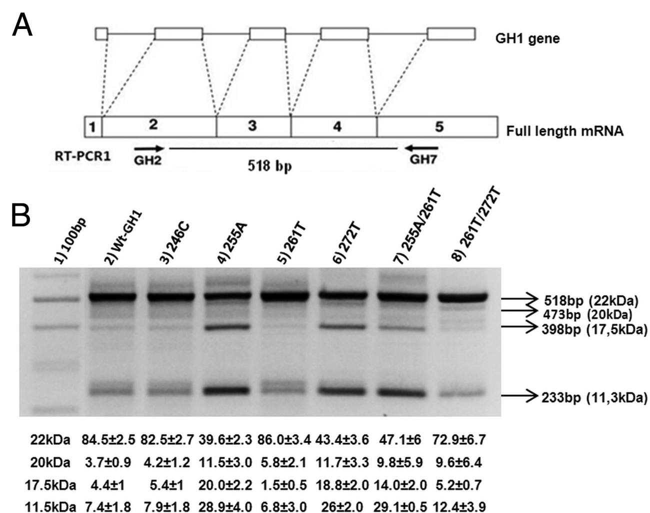

Figure 2. A, Scheme of GH1 pre-mRNA splicing showing the full-length mRNA and the corresponding RT-PCR product. B, RT-PCR performed on mRNA extracted from GH4C1 pituitary cells transfected with the wild-type *GH1* (lane 2) or constructs carrying different variations (lanes 3–8). The size of the bands are indicated on the right with the corresponding protein molecular weight of the different isoforms. Untransfected rat cDNA did not show any bands (data not shown). Each PCR product was eluted from the gel and characterized by sequencing. The relative band intensity (expressed as the percentage on the total yield) calculated as the mean ± SD over 4 different independent transfection experiments is reported for each construct below the corresponding lane.

ESE finder analysis

Analysis of the splicing regulatory motifs within exon 3 was performed using the software ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi). The default thresholds were considered to identify sites responsible for the 4 serine/arginine-rich (SR) proteins alternative splicing factor 1/pre-mRNA splicing factor SF2 (ASF/SF2), serine/arginine-rich splicing factor 3 (SC35), serine/arginine-rich splicing factor 5 (SRp40), and serine/arginine-rich splicing factor 6 (SRp55).

Gel image analysis

The RT-PCR and Western blotting gel images were analyzed using the freeware ImageJ1.46r (http://rsb.info.nih.gov/ij/; National Institutes of Health), and the bands were quantified by measuring pixel intensity and normalized to the corresponding β-actin band intensity.

Results

Genetic analysis of the IGHD patients

A total of 103 sporadic IGHD patients with height ranging from −1.8 to −4.5 SDS were investigated for the presence of functionally relevant mutations in *GH1*, including coding regions and introns. Two patients harbored a combination of 2 variations within exon 3 (Table 1) that were not previously reported in public databases, including dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), the Exome Variant Server (http://evs.gs.washington.edu/EVS/), and 1000 genomes (http://www.1000genomes.org/). Patient 1 carried c.261C>T (p.Pro87Pro) and c.272A>T (p.Glu91Val), and patient 2 carried c.255G>A (p.Pro85Pro) and c.261C>T (p.Pro87Pro).

A third patient (patient 3) (Table 1) carried the nonsynonymous c.246G>C determining the substitution p.Glu82Asp in exon 3. This variation was reported in the dbSNP (rs61762497) only in 1 individual and in the Exome Variant Server in 2 out of 13 000 individuals.

All the above exon 3 variations were absent in a panel of 410 chromosomes sequenced from 205 normal stature Italian control individuals.

The analysis of the parents of patients 2 and 3 demonstrated that their variants were inherited from the unaffected fathers. Unfortunately, the parents of patient 1 did not give their consent to DNA analysis. By subcloning the patient's PCR products in a TA cloning vector system, we confirmed that in patient 1, as well as in patient 2, both

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variants were on the same allele. The alignment of *GH1* with the paralogous genes of the GH cluster suggested that all the 4 variants in exon 3 were generated by nonallelic gene conversion from the *GH2* gene, because the other 3 genes (*CS-5*, *CS-2*, and *CS-1*) have the same *GH1* sequence at these sites.

The exon 3 variations fall within predicted ESEs

Because several exon 3 mutations lead to missplicing of mRNA and production of increased amounts of the 17.5-kDa protein, we evaluated the possible involvement in the splicing regulation of the variants identified in our patients within this exon. None of them was included in the previously described ESEs (ESE1 and ESE2). An in silico analysis using ESE finder 3.0 (Figure 1, A and B) revealed the presence of 2 high scoring ESE motifs from c.254 to c.260 (CGACACC) and c.269 to c.275 (GGGAGGA). These 2 sequences include the variations c.255G>A and c.272 A>T, respectively (Figure 1A). The position c.255 is located within an ESE motif recognized by the SR protein SRp40 and c.272 within a sequence recognized by SRp40 and SF2/ASF. Both putative ESE sequences showed an increased score compared with the threshold value (Figure 1B). By substituting the wild-type nucleotides with those found in the patients, namely c.255A and c.272T, the software predicted the complete loss of these 2 putative ESEs (Figure 1B).

Variants c.255A and c.272T affect *GH1* splicing in vitro

To evaluate whether exon 3 variants actually had some effect on mRNA splicing in vitro, we transfected GH4C1 rat pituitary cells with an expression vector containing 1) the wild-type allele (wt-*GH1*), 2) the alleles carrying the single mutations, and 3) the alleles containing the mutations combined as they were in patients 1 and 2. The mRNA from transfected cells was reverse transcribed, and the resulting cDNA was amplified with primers (Figure 2A) specific for the human *GH1* that did not amplify the rat mRNA. The RT-PCR on the wt-*GH1* mRNA (Figure 2B, lane 2) yielded an intense band corresponding to the *GH1* full-length transcript (518 bp) producing the 22-kDa protein and faint bands corresponding to the 20-kDa isoform (473 bp), the 17.5-kDa isoform (398 bp), and the 11.3-kDa isoform (233 bp). The average yield of the mRNA corresponding to the 17.5-kDa isoform over 4 independent experiments was 4.4 ± 1% of the total mRNA yield with a ratio 17.5 kDa/22 kDa of about 1:20. The 246C construct (Figure 2B, lane 3) was also tested, although the c.246C variation was not predicted to influence splicing. This construct generated a band pattern similar to that observed in the wild-type accordingly to the in silico prediction. In contrast, 255A and 272T constructs (Figure 2B, lanes 4 and 6, respectively) produced a higher level of the exon 3-skipped mRNA with a ratio 17.5 kDa/22 kDa of about 1:2 and 1:2.4, respectively. In these 2 constructs, there was also an evident increased production of the exon 3–4-skipped transcript (233-bp band). Interestingly, the exon 3-skipped mRNA was not evident in the transcripts from the 261T construct, suggesting that this variant might strengthen the correct splicing. When the variants were combined on the same construct to reproduce the status of patients 1 and 2, the exon 3-skipped mRNA produced by the construct 255A/261T (patient 2) (Figure 2B, lane 7) was less abundant than that observed for 255A but still evident, with a 17.5 kDa/22 kDa ratio of 1:3.3. Conversely, the 261T/272T construct (patient 1) (Figure 2B, lane 8) showed a splicing pattern very similar to the wild type.

We then investigated the consequences of the variants on protein synthesis (Figure 3). Western blot analysis confirmed that the transfected wt-*GH1*-produced mostly the 22-kDa protein with only traces of the 20 and 17.5 kDa. A higher level of the 17.5-kDa product was produced by 255A, 272T, and by the 255A/261T constructs. Although the 261T/272T plasmid carrying the 2 variations detected in patient 1 showed a mRNA pattern similar to the wild type (Figure 2B, lane 8), it exhibited a band in correspondence of the 17.5-kDa protein more intense than the wild type (about 10% of the total GH proteins) (Figure 3, lane 7). This band in the 261T construct was weak (as in the wt-*GH1*), but the 20-kDa isoform was more intense than in all the other constructs. The corresponding 11.3-kDa protein isoform was not detectable by the antibody used for Western blot analysis.

Discussion

In the present study, we sequenced *GH1* in 103 IGHD patients with clinically variable phenotypes and no family

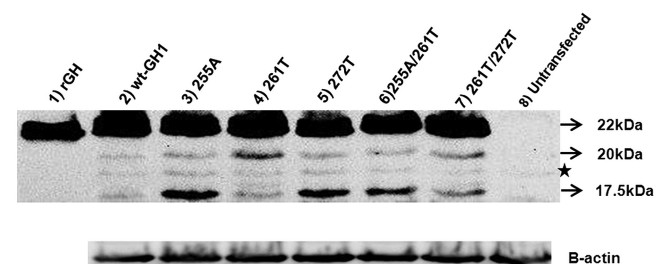

Figure 3. Western immunoblot analysis of the different GH isoforms encoded by the different splicing products. The CHO cells were transiently transfected with either wt-*GH1* (lane 2) or constructs carrying different variations (lanes 3–7). Untransfected CHO cells were used as a negative control (lane 8); *, nonspecific bands also present in the untransfected CHO cell lysate.

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history to search for mutations that might be 1) inherited from healthy parents (incomplete penetrance), 2) arisen de novo, or 3) biallelic (recessive inheritance). We identified 3 patients that carried variations in exon 3, and 2 of them carried 2 variants on the same allele. All the variants were likely generated by gene conversion, an event that is frequently associated to *GH1* sequence variability (19–21). An in silico analysis was performed using ESE finder 3.0, which has been designed to identify SR protein binding sites by generating a motif score that reflects the binding site strength. The sequences from c.254 to c.260 and from c.269 to c.275, including the positions c.255 and c.272 mutated in patient 2 and in patient 1, respectively, were predicted to represent ESE motifs recognized by the SRp40 and SF2/ASF proteins with a score above the threshold (Figure 1B). The same factors were predicted to recognize ESE2 with the same strength (9). It has been demonstrated that SF2/ASF activates exon 3 inclusion and that disruption of this motif causes increased exon 3 skipping (22). The substitution of the wild-type nucleotides c.255G and c.272A with the mutants c.255A and c.272T, respectively, was predicted to abolish the binding of these proteins in both putative ESEs (Figure 1). The RT-PCR analysis (Figure 2) confirmed the influence of c.255A and c.272T on splicing by increasing the exon 3-skipped isoform to 18%–20% of the total GH mRNA (Figure 2B, lanes 4 and 6, respectively).

Mutations within ESE1 in *GH1* have been previously reported to cause either complete or partial exon 3 skipping and generation of increased amounts of the 20- and the 17.5-kDa isoforms at various concentrations (20%–37% and 35%–68%, respectively) (8, 9). The clinical variability observed in patients carrying these mutations has been explained by variable amounts of the 17.5-kDa isoform consequent to a weakened exon 3 recognition. Hamid et al (23) reported a large pedigree with the c.172G>A splicing mutation, and they suggested that the ratio of 17.5 kDa/22 kDa transcripts in the lymphocytes correlated with the height SDS before GH replacement therapy. In this family, there were individuals with a height SDS more than −2 that inherited the mutation (incomplete penetrance). Another heterozygous missense mutation, c.200A>G, within ESE2 induces exon skipping in about 20% of the transcripts, giving rise to different phenotypes ranging from short stature to normal stature in the same large pedigrees (19).

It is thus conceivable that mutations associated with variable expressivity and incomplete penetrance might be responsible for at least some milder forms of IGHD.

The effect determined by the here detected variants, c.255G>A and c.272 A>T, is comparable with the effect caused by most of the previously described mutations falling within ESE1 and ESE2 (8, 19, 23). However, when these 2 variants were combined with c.261T, as in patients 1 and 2, the effect on splicing was maintained, although weaker, only for the 255A/261T construct (patient 2) (Figure 2B, lane 7). In contrast, the 261T/272T construct (patient 1) (Figure 2B, lane 8) was very similar to the wild-type, although the Western blotting for the same construct showed a slightly increased amount of the 17.5-kDa band (Figure 3, lane 7). This discrepancy might be attributable to the different sensitivity of the two methods.

Thus, the c.261 variant, which is not included in the putative ESE sites, partially hides the negative effect of the c.272. It can be speculated that c.261 influences splicing by strengthening the affinity for other proteins involved in the correct splicing regulation.

It is worth considering that the 233-bp band corresponding to the bioinactive 11.3 kDa (exon 3–4-skipped isoform) is strongly increased in all mutant constructs, but 261T and 261T/272T (Figure 2B). The relative total amount of the 2 alternatively spliced mRNA (exon 3 and exon 3–4 skipped) in the 255A, 272T, and 255A/261T constructs can be roughly estimated to represent nearly 50% of the total *GH1* mRNA in contrast to the wild-type, where these transcripts represent about 12% of the *GH1* transcripts.

The low serum GH level detected in vivo in the patients might be in part determined by the effect on splicing of these variants and in part by other yet unidentified genetic factors. Notably, patient 1 carried on the other allele the *GH1* promoter haplotype 1 (data not shown) that has been associated to IGHD and to a reduced luciferase activity in vitro (24).

The functional significance of the Glu82Asp variant carried by patient 3 is uncertain. From our experiments, it does not seem to influence splicing (Figure 2B, lane 3), and it is not predicted to have an effect on SR protein binding (data not shown). This variant is very rare, because it was reported in the dbSNP database (rs61762497) only in 1 individual of African ancestry, it is present in 2 individuals out of 13 000 of the Exome Variant Server, and it was absent in our panel of 205 normal stature individuals. However, it is not predicted to exert a damaging effect on the protein function by the software Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). It might, thus, represent either a rare benign polymorphism, or alternatively it might contribute to a multigenic form of IGHD in this patient.

In conclusion, the analysis of *GH1* in individuals with sporadic IGHD led to the identification of 2 novel *GH1* exon 3 variations, c.255 G>A and c.272 A>T, included within 2 novel putative splicing regulatory elements that increase the aberrant splicing in vitro. When combined in

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cis, with c.261 C>T, as in the patients, their effect was reduced but still evident on the protein synthesis. It can be hypothesized that also a minimally increased amount of the 17.5-kDa protein might exert a dominant negative effect on the GH synthesis in vivo. The phenotype of patients 1 and 2 might be associated to these *GH1* splicing variations that by themselves only partially influence the amount of GH secretion but that might act in concert with other genetic variants.

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