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Genetics

Alternative splicing of exon 3 of the human growth hormone receptor is the result of an unusual genetic polymorphism

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ABSTRACT **Two isoforms of the human growth hormone receptor (hGHR), which differ in the presence (hGHRwt) or absence (hGHRd3) of exon 3, are expressed in the placenta. Specifically, three expression patterns are observed: only hGHRwt, only hGHRd3, or an approximately 1:1 combination of both isoforms. We investigated several potential regulatory mechanisms which might account for the expression of the hGHR isoforms. The frequency of hGHRd3 expression did not change when placentas from differing stages of gestation were examined, suggesting splicing was not developmentally regulated. However, when hGHR isoform expression patterns were examined in each component of a given placenta, it was evident that alternative splicing of exon 3 is individual-specific. Surprisingly, the individual-specific regulation of hGHR isoforms appears to be the result of a polymorphism in the hGHR gene. We analyzed hGHRwt and hGHRd3 expression in Hutterite pedigrees, and our results are consistent with a simple Mendelian inheritance of two differing alleles in which exon 3 is spliced in an "all-or-none" fashion. We conclude the alternative splicing of exon 3 in hGHR transcripts is the result of an unusual polymorphism which significantly alters splicing of the hGHR transcript and that the relatively high frequency (≈10%) of homozygous hGHRd3 expression suggests the possibility it may play a role in polygenic determined events.**

In humans, chorionic somatomammotropin (hCS) and growth hormone (hGH) are encoded by a family of genes clustered on chromosome 17 (1). At least two forms of each hormone are encoded by separate genes for both hCS (hCS-A and hCS-B) and hGH (hGH-N and hGH-V). hGH-N, which is expressed only in the anterior lobe of the pituitary, is best known for its effects on the growth of skeletal and soft tissues and for its metabolic actions. The remaining proteins are expressed exclusively in the placenta. Their precise function remains obscure, and the picture is further complicated by the presence of alternatively spliced mRNAs that can give rise to additional isoforms.

The synthesis and secretion of hCS, hGH-V, and their multiple isoforms in the placenta raises an important question as to whether a single receptor mediates the activity of these proteins or if different receptors exist. A receptor for hGH (hGHR) has been cloned (2) and shown to belong to the cytokine receptor family (3). A second, alternatively spliced, form of hGHR lacking exon 3 (hGHRd3) was recently identified from a placental cDNA library (4). It has been suggested that exclusion of exon 3 results in an hGHR with structural features more characteristic of a prolactin receptor and that exon 3 may be important in differentiating somatogen from lactogen receptors within the placenta (4). Although the binding affinities of hGHR containing exon 3 (hGHRwt) and hGHRd3 for ligand (hGH-V or hCS) are indistinguishable (5, 6), functional differences at the level of signal transduction may exist.

The mode of regulation for the alternative splicing of hGHR is unclear. It was originally reported that hGHRd3 expression was limited to placental villi and amnion (4, 6), whereas hGHRwt was found only in chorion and decidua (4). However, both isoforms were observed in normal adult hepatic tissue and in fetal and cancerous liver samples, indicating isoform expression was not tissue-specific or developmentally regulated (7). Likewise, both isoforms were expressed in 19 different tissues obtained from autopsied individuals (8). Comparison of hGHR isoform expression patterns in tumor biopsies and lymphocytes taken from the same patient indicated individual-specific regulation (9). Similarly, Wickelgren *et al.* (10) found that the expression patterns of hGHR varied among individuals, but different tissues from the same subject showed the same expression pattern. Finally, expression of hGHR isoforms in placentas of differing stages was examined and, in contrast to the liver, splicing was shown to be developmentally regulated and individual-specific (11).

The placenta is unique in that it expresses multiple proteins and their isoforms of the GH/CS family, and we felt that a more thorough characterization of hGHR isoform expression was warranted. Surprisingly, we find that the skipping of exon 3 in hGHR is not the result of regulated pre-mRNA splicing. Instead, it appears to be an unusual genetic polymorphism which results in total exclusion of an entire exon without affecting any of the sequences within pre-mRNAs commonly known to affect splicing.

MATERIALS AND METHODS

Tissues and RNA Extraction. Term placentas (36–42 weeks) were obtained after delivery at University Hospitals (Cleveland, OH) under a protocol approved by the Institutional Review Board for Human Investigation. Villous tissue was obtained from all placentas, and in some cases the placentas were further dissected into amnion, chorionic plate, and decidua. For prematurely delivered placentas (20–36 weeks), a small piece (1 g) was obtained immediately after delivery and the villi and decidua were removed for use in these studies. Chromosomally normal, spontaneously aborted trophoblast tissue (11–20 weeks) was kindly provided by T. Hassold (Case Western Reserve University). Blood samples were obtained from large, multigenerational Hutterite families and lymphoblastoid cell lines were established by transformation with Epstein–Barr virus, as previously described (12). Total cellular RNA was extracted from tissue or cells using the TRIzol reagent (GIBCO/BRL) according to the manufacturer's instructions.

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Abbreviations: hGHR, human growth hormone receptor; hGHRwt, human growth hormone receptor (exon 3 included); hGHRd3, human growth hormone receptor (exon 3 deleted); PlGF, placenta growth factor; hCS, human chorionic somatomammotropin; hGH-V, human growth hormone variant; RT-PCR, reverse transcription–polymerase chain reaction.

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Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Southern Hybridization. Approximately 1.5 μg of total RNA was denatured at 94°C for 3 min. Reverse transcriptase buffer (250 mM Tris·HCl, pH 8.3/375 mM KCl/15 mM $MgCl_2$) was added and the reaction was carried out for 1 h in the presence of 66 ng of antisense primer (481a, 5′-GGTTG-CACTATTTCCATCAAC-3′), 1 mM dNTPs, 20–40 units of RNAsin (Promega), and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) at 42°C or 200 units of SuperScript II reverse transcriptase (GIBCO/BRL) at 44°C. The reverse transcription reaction was terminated by heating for 5 min at 95°C. PCR was performed using 4 μl of the reverse transcription reaction product in PCR buffer (200 mM Tris·HCl, pH 8.4/500 mM KCl) containing 2.0 units of *Taq* DNA polymerase (GIBCO/BRL or United States Biochemical), 0.8 mM dNTPs, and 40 ng of sense (41, 5′-GGT-ATGGATCTCTGGCAG-3′) and antisense (481a) primers. PCR was carried out for 30–35 cycles on a Hybaid OmniGene Thermocycler (Holbrook, NY). In the initial cycle, the denaturation step was extended to 5 min, during which the *Taq* DNA polymerase was added. Subsequent cycles were as follows: denaturation 94°C, 30 sec; annealing 62°C, 30 sec; and elongation 72°C, 60 sec. As a control for the PCR portion of the assay, a separate reaction was included in all experiments in which a cDNA clone containing hGHRd3 (kindly provided by J. Kopchick, Edison Biotechnology Institute, Ohio University) was used as the template DNA. The same conditions were used to amplify placenta growth factor (PlGF) using specific primers (PlGF-1, 5′-CTCCTAAAGATCCGTTCTGG-3′; PlGF-2, 5′-GGTAATAAATACACGAGCCG-3′; ref. 13) with an annealing temperature of 54°C in the PCR.

For Southern hybridization, DNA fragments were transferred to a nylon membrane (Zeta Probe, Bio-Rad) in a Trans-Blot SD transfer cell (Bio-Rad). The blots were prehybridized 5 h or overnight at 42°C (2× SSC/1× Denhardt's solution/0.1% SDS/0.5 mg of sonicated herring sperm DNA per ml), then hybridized overnight at 42°C in the same buffer containing a ^{32}P-end-labeled oligonucleotide probe (hGHR, 303a, 5′-CTTGAGTGTTCCTTCTGG-3′; PlGF-2). Afterwards, the filters were washed at 42°C with two changes of buffer (0.1× SSC/0.1% SDS) and exposed to x-ray film (XRP or XAR, Eastman Kodak) for 6–24 h at −80°C in the presence of intensifying screens.

Subcloning and Sequence Analysis of Exons 2, 3, and 4 and Adjacent Introns. Genomic DNA was isolated from the placenta of 10 individuals by using the TRIzol reagent according to the manufacturer's instructions. Oligonucleotide primers complementary to sequences in adjacent introns (hGHR2-F, 5′-TTCAAGCTTATGGATCTCTGGCAGC-3′; hGHR2-R, 5′-CCTGGATCCAGTTCAGTGTTG-3′; hGHR3a-F, 5′-GA-GGTCTATTTAGTCAGTCTTATG-3′; hGHR3b-F, 5′-CC-TGGATCCACAGGGTCATATCAGN-3′; hGHR3-R, 5′-GGGATAGTAGCTTAATTACAC-3′; hGHR4-F, 5′-CCC-ATCACATATGACTCACCT-3′; and hGHR4-R, 5′-CTTG-AATTCTGGTATAGAACAGC-3′) were used to amplify exons 2, 3, and 4 from genomic DNA. Only a small portion of the two introns (total length = >14 kbp and 20 kbp, respectively) have been sequenced (see ref. 14), and the primers used in these studies were designed to include as much intron sequence in the amplified products as possible. The amplified products were subcloned into pBluescript II (±) plasmids (Stratagene). Plasmid inserts were sequenced by the dideoxynucleotide method (15) with M13 forward primer (16) and internal primers (hGHR2-R, hGHR3-R, and hGHR4-R, see above).

RESULTS

Expression of hGHR mRNA Isoforms in Placental Villi. Villous tissue was obtained from 15 term placentas and examined for hGHR isoform content by RT-PCR analysis. Fig. 1*A* shows the strategy for analysis of exon 3 expression in hGHR, and representative samples are shown in Fig. 1*B*. In 2

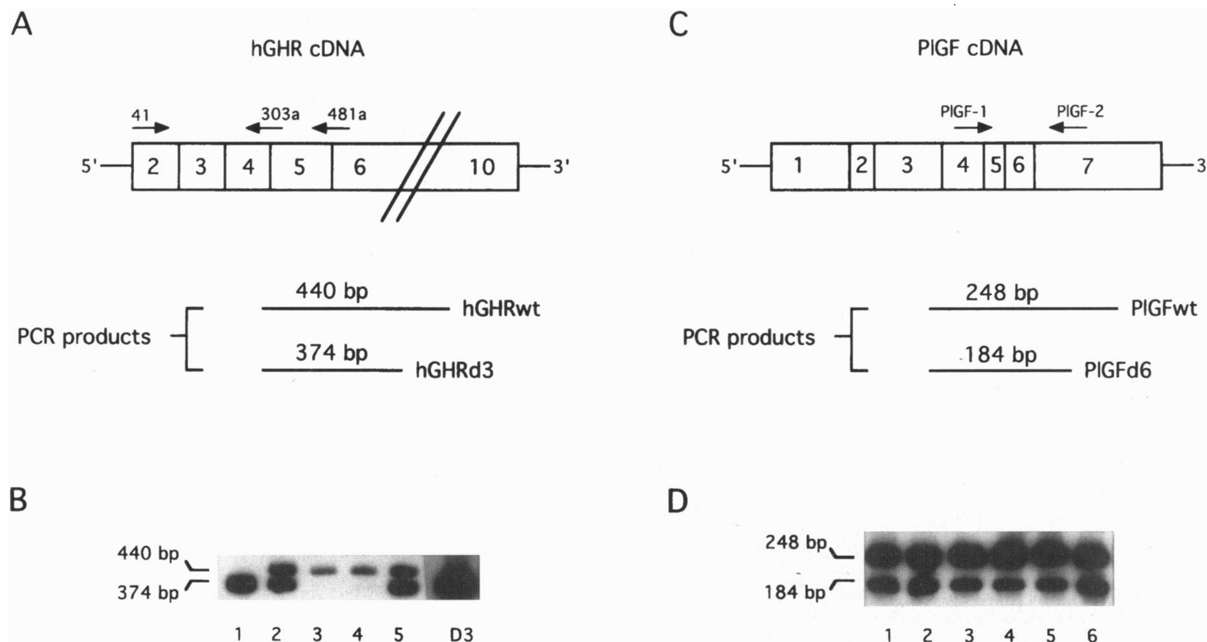

FIG. 1. Expression of hGHR and PlGF isoforms in placental villi. (*A*) Strategy for analysis of exon 3 expression in hGHR. The exons in the hGHR coding region are represented by boxes, and solid lines represent the 5′ and 3′ untranslated regions. The positions of the oligonucleotides used in this experiment are shown as horizontal arrows. The predicted lengths of the PCR products are given in base pairs (bp). (*B*) RT-PCR analysis of hGHR isoform expression patterns in the villi of term placentas. An autoradiogram of the Southern blotted products from five representative placentas (lanes 1–5) and an hGHRd3 plasmid (lane 6) are shown. (*C*) Strategy for analysis of exon 6 expression in PlGF. The exons in the PlGF coding region are represented by boxes, and solid lines represent the 5′ and 3′ untranslated regions. The positions of the oligonucleotides used in this experiment are shown as horizontal arrows. The predicted lengths of the PCR products are given in bp. (*D*) RT-PCR analysis of PlGF isoform expression patterns in the villi of term placentas. An autoradiogram of the Southern blotted products from six representative placentas (lanes 1–6) is shown.

of the 15 placentas examined, a single band corresponding to hGHRwt was detected (no evidence of hGHRd3 expression, even after prolonged exposure of the autoradiograph). Ten samples contained both forms of hGHR, and the remaining 3 placentas contained only hGHRd3. In the samples in which both isoforms appeared, semiquantitative analysis by densitometry indicated almost equal amounts of each (45% hGHRwt and 55% hGHRd3).

The unexpected variation in the expression patterns observed for hGHR in our samples indicated that the inclusion or exclusion of exon 3 was not tissue-specific. As a control, the alternative splicing of another placental protein, PlGF, was also examined. Exon 6 had previously been shown to be excluded (PlGFd6) in a small proportion of the PlGF transcripts in term villi samples (13). Fig. 1*C* shows the strategy for analysis of exon 6, and representative samples of PlGF expression are shown in Fig. 1*D*. In all placental villi samples examined, both isoforms were detected at a ratio of approximately 80% PlGFwt to 20% PlGFd6, which is in accordance with previous studies (13). Thus, the variable expression of hGHR isoforms among individuals did not appear to be the result of a general disruption in the splicing machinery in the placenta.

Is Alternative Splicing of Exon 3 Affected by Gestational Age? It was clear from the previous experiment that alternative splicing of exon 3 from hGHR was variable, but the mode of control was not obvious. Since it had recently been proposed that exclusion of exon 3 from hGHR mRNAs was under developmental control in the placenta (11), 75 placentas were obtained and divided into the following gestational age groups: <20 wk (n = 15), 20–29 wk (n = 8), 30–36 wk (n = 21), and 37–41 wk (n = 30). RT-PCR analysis of hGHR isoform content in villi did not reveal any differences in expression patterns among gestational age groups (Table 1). Coexpression of hGHRwt and hGHRd3 was the most common pattern of hGHR isoforms observed, and in all cases both isoforms were coexpressed at approximately equal levels. Expression of only hGHRd3 was rare and accounted for <10% of the total placentas examined. Developmental control of hGHR mRNA splicing was clearly not indicated from this study.

Is Alternative Splicing of Exon 3 Individual-Specific? Previous studies indicate splicing of exon 3 may be individual-specific (9–11). To investigate this possibility, 24 term placentas were dissected into the fetal (villi, amnion, chorion) and maternal (decidua) components. The hGHR mRNA isoform content, determined by RT-PCR analysis, in each of the fetally derived tissues was always identical in any given placenta (Fig. 2, Table 2). However, expression of hGHR mRNA isoforms in the maternally derived decidua was not always the same as in the remaining placental components. It was clear from this experiment that splicing of exon 3 was regulated differently in the maternally derived tissues compared with the fetally derived tissues. Thus, our data support the hypothesis that alternative splicing of hGHR is individual-specific.

Is the Individual-Specific Control of hGHR mRNA Splicing the Result of Allelic Differences? Wickelgren *et al.* (10) suggested that the individual-specific control of hGHR isoform expression was under the control of factors that affect all tissues in the body. However, it seemed odd that exon-skipping in the hGHR transcript but not other mRNAs would be affected. We observed from the previous experiment that certain combinations of isoform expression patterns in the fetal and maternal tissues were not detected. Specifically, expression of only hGHRwt in the fetal tissues was never accompanied by expression of only hGHRd3 in the decidua of that same placenta. Conversely, fetal expression of only hGHRd3 combined with expression of only hGHRwt in the decidua was not observed within any given placenta. Since our sample size was small, we also examined maternal and fetal expression of hGHR isoforms in placentas (n = 59) from the gestational age study. By RT-PCR analysis, 31 placentas were identified with 100% hGHRwt expression in the villi (Table 3). In the decidua from these same placentas, the only isoform expression patterns detected were 100% hGHRwt or coexpression of both forms. Six placentas were identified with 100% hGHRd3 expression in the villi, but decidual expression was limited to either 100% hGHRd3 or coexpression of both forms. In the remaining placentas (n = 43), coexpression of both forms occurred in the villi, and all three expression patterns of hGHR isoforms appeared in the decidua. A placenta with 100% hGHRwt expression in the villi and 100% hGHRd3 expression in the decidua, or the reverse situation of 100% hGHRd3 expression in the villi and 100% hGHRwt expression in the decidua, was not observed. We came to the unexpected conclusion that the individual-specific control of exon 3 splicing might be the result of an allele difference. In other words, hGHRd3 might represent an allele in which the entire exon 3 was deleted or in which a mutation has arisen that would render the splicing of exon 3 completely inoperable. This model for exon 3 splicing also assumes the hGHRwt and hGHRd3 alleles are codominant, based on our observation that the isoforms are expressed in almost equal amounts (45% hGHRwt and 55% hGHRd3) in those samples in which both appear.

To exclude the possibility that splicing of exon 3 was influenced by maternal factors rather than allelic differences, lymphoblastoid cell lines originating from five Hutterite pedigrees were examined for expression of hGHR isoforms by

Table 1. Abundance of hGHR isoform expression patterns in villous tissue of placentas of differing gestational ages

		No. of placentas		
Age, wk	n	100% hGHRwt	hGHRwt and hGHRd3	100% hGHRd3
37–41	30	12	16	2
30–36	21	6	12	3
20–29	8	3	5	0
<20	15	6	8	1

Table 2. Expression of hGHR isoforms in placental components

	Expression					
Component	n = 12	n = 4	n = 5	n = 1	n = 1	n = 1
Fetal						
Villi	WT	B	B	WT	D3	B
Chorion	WT	B	B	WT	D3	B
Amnion	WT	B	B	WT	D3	B
Maternal						
Decidua	WT	WT	B	B	D3	D3

WT, 100% expression hGHRwt; D3, 100% expression hGHRd3; B, coexpression of hGHRwt and hGHRd3.

Table 3. Expression of hGHR isoforms in fetal (villi) and maternal (decidua) placental components

Villi	Decidua	No. of placentas
WT	WT	24
	B	7
	D3	0
B	WT	15
	B	24
	D3	4
D3	WT	0
	B	2
	D3	4

WT, 100% expression hGHRwt; B, coexpression of hGHRwt and hGHRd3; D3, 100% expression hGHRd3.

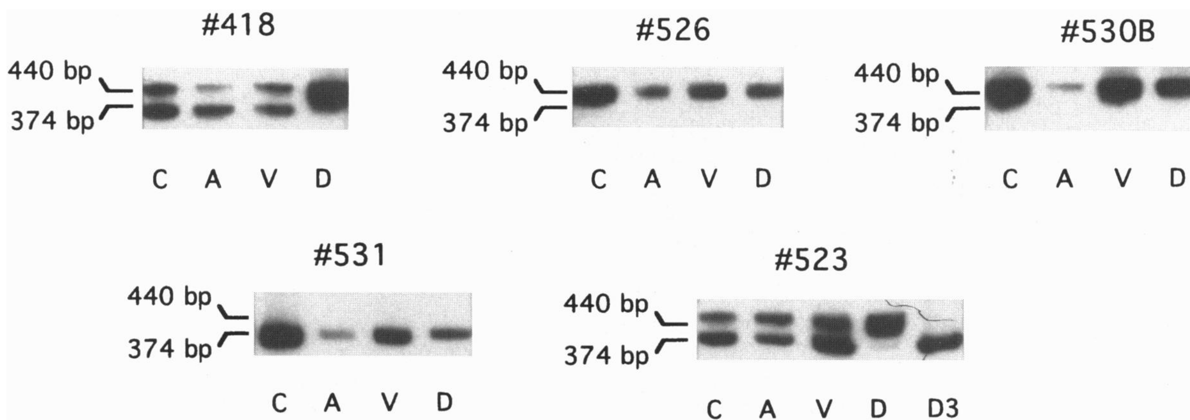

FIG. 2. RT-PCR analysis of hGHR isoforms in placental tissues. Autoradiograms of the Southern blotted products from five representative placentas are shown. C, chorion; A, amnion; V, villi; D, decidua; D3, hGHRd3 plasmid.

RT-PCR analysis. Most of the families involved an hGHRwt individual (male or female) married to an individual who coexpressed hGHRwt and hGHRd3 (Fig. 3). The expected hGHR isoform expression patterns in the offspring are hGH-Rwt (50%) and coexpression of both isoforms (50%). The observed phenotypes (hGHRwt, $n = 15$; coexpression of hGHRwt and hGHRd3, $n = 8$) did not differ significantly from the expected phenotypes ($\chi^2 = 2.13, P > 0.10$). The remaining pedigree involved a family in which each parent coexpressed both isoforms. The distribution of expected expression patterns from this marriage would be hGHRwt (25%), coexpression of both forms (50%), and hGHRd3 (25%). Unfortunately, only a limited number of offspring were available but the resulting phenotypes (hGHRwt, $n = 1$; coexpression of hGH-Rwt and hGHRd3, $n = 2$) were consistent with a simple Mendelian type of inheritance. Thus, the individual-specific mode of regulation for splicing of exon 3 from hGHR transcripts appears to be due to allelic differences.

Are the 5′ and/or 3′ Splice Sites in the Introns Surrounding Exon 3 Mutated in hGHRd3 Individuals? It was of interest to determine if a mutation(s) that might cause exon 3 to be skipped existed either within or directly adjacent to the 5′ and 3′ splice sites of the introns flanking exon 3 in the hGHR gene in those individuals expressing 100% hGHRd3. Genomic DNA was isolated from 10 individuals expressing 100% hGH-Rwt, 100% hGHRd3, or both isoforms. Exons 2, 3, and 4 and extended regions of adjacent introns were amplified from the DNA, using primers designed to include all elements of the splice sites. The sequence analyses of the exons are presented in Fig. 4. DNA fragments corresponding to exon 3 were amplified from all individuals, indicating the hGHRd3 phenotype is the result of a splicing event rather than a genomic deletion. Point mutations were detected in the intron sequences near exon 2 and exon 4 (data not shown), but these mutations did not correlate with the hGHRd3 phenotype. Deletions of 1 or 2 bp were identified in the 3′ end of the intron located between exons 2 and 3, but these were present in individuals of all phenotypes. Sequencing of an additional 200 bp in this intron immediately 5′ to the branch point also failed to reveal any significant differences between individuals with different expression patterns (data not shown). Although numerous mutations, some which result in nonconservative amino acid substitutions, were detected in a short sequence within exon 4, there was no correlation with the hGHRd3 phenotype. Thus, it appears that the allele differences that result in exclusion of exon 3 likely occur in other portions of the hGHR gene.

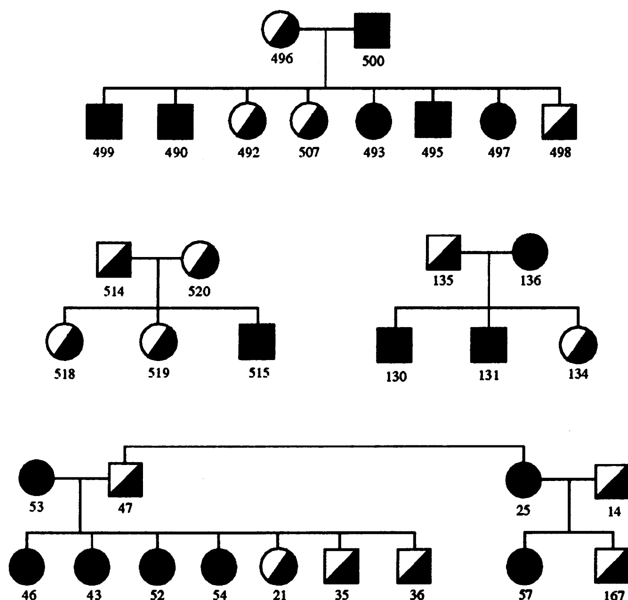

FIG. 3. Pedigree analysis of hGHR isoforms in four representative Hutterite families. The solid symbols represent individuals with only hGHRwt expression and the partially filled symbols represent coexpression of hGHR isoforms. Individuals expressing only hGHRd3 were not identified in these families. The squares denote males and the circles represent female family members.

DISCUSSION

We felt it was important to reexamine placental expression of hGHR mRNA isoforms because the placenta synthesizes and secretes several potential hGHR ligands, including hCS-A, hCS-B, and hGH-V. The receptor for hGH-N has been identified in the placenta, but it is unknown if hGHR mediates the activity of hCS or hGH-V. Therefore, identification of an hGHR in the placenta lacking exon 3 was very exciting, since there was a possibility that it might function in differentiating somatogen from lactogen receptors, a potentially important role for placental and fetal development (4).

We observed essentially three expression patterns of hGHR isoforms: only hGHRwt, only hGHRd3, or coexpression of each at similar levels. The variable expression of hGHR isoforms indicated an influence on splicing, but it was difficult to correlate the appearance of the splicing patterns with any known parameters. Esposito *et al.* (7) suggested that the exclusion of exon 3 was random in nature, but we found it difficult to comprehend that an exon would be alternatively spliced in an uncontrolled fashion.

Developmental regulation of alternative splicing is common, and it has been implicated in the control of exon 3 splicing for

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                                 EXON 2
                           exon      intron
                                                   *
Consensus                  5'GAGGgtgagttctgcttttccatttcca3'
Individual #2    +/+       5'GAGGgtgagttctgcttttccatttcca3'
Individual #1    +/-       5'GAGGgtgagttctgcttttccatttcca3'
Individual #3    +/-       5'GAGGgtgagttctgcttttccattccca3'
Individual #5    -/-       5'GAGGgtgagttctgcttttccatttcca3'
Individual #8    -/-       5'GAGGgtgagttctgcttttccattccca3'

                                 EXON 3
                            intron                      exon
                          *        **    *
Consensus                5'agatggttttgccttcct...cagCCA...CAAgta3'
Individual #2 +/+        5'a-atggtttt--ctt-ct...cagCCA...CAAgta3'
Individual #1 +/-        5'a-atggtttt--ctt-ct...cagCCA...CAAgta3'
Individual #8 -/-        5'a-atggtttt--ctt-ct...cagCCA...CAAgta3'

                                 EXON 4
                                   exon
                                    *          *         *
Consensus                5'agATT...CCGTT...GAGAGACTTTTTCATGCCAC3'
Individual #2 +/+        5'agATT...CCGTT...GAGAGACCTTTTCATGCCAC3'
Individual #7 +/+        5'agATT...CCGTT...GAGAGACTTTTTCATGCCCC3'
Individual #1 +/-        5'agATT...CCATT...GAGAGACCTTTTCATGCCAC3'
Individual #3 +/-        5'agATT...CCGTT...GAGAGACTTTTTCATGCCAC3'
Individual #6 +/-        5'agATT...CCGTT...GAGAGACCTTTTCATGCCAC3'
Individual #5 -/-        5'agATT...CCGTT...GAGAGACTTTTTCATGCCAC3'
Individual #8 -/-        5'agATT...CCGTT...GAGAGACCTTTTCATGCCAC3'
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FIG. 4. Sequence analysis of the hGHR gene. Genomic DNA was obtained from the villous portion of placentas from a number of individuals expressing only hGHRwt (+/+), both isoforms (+/−), or only hGHRd3 (−/−). The regions of the two introns flanking exon 3, including the 5′ splice donor and 3′ splice acceptor sites, are shown. Additionally, a short region found within exon 4 was included, since numerous mutations were identified in that portion of the gene. Mutations are underlined and denoted by asterisks. The exonic portions of the sequence are shown capitalized and in boldface type.

hGHR. Zogopoulos *et al.* (11) examined hGHR isoform expression patterns in fetal and postnatal tissues and found that major expression of hGHRd3 occurred prior to midgestation. Since hGH does not appear to be critical for prenatal growth, it was suggested that hGHRd3 might represent an "immature" form of the receptor and that abundant expression of this isoform during gestation could explain why hGH does not play a role in fetal growth (11). Although this hypothesis was interesting, differences in the frequency of hGHRd3 expression during early, mid, or late gestation were not found in our samples.

Recently, it has been proposed that expression of hGHR isoforms is regulated in an individual-specific manner. Our observation that isoform expression patterns were always identical in the fetal components of a given placenta but sometimes different in the maternally derived decidua were consistent with individual-specific regulation of hGHR alternative splicing. Wickelgren *et al.* (10) suggested the splicing of exon 3 was under the control of factors that affect all tissues in the body, but it was difficult to understand how cellular trans-acting factors might be involved. Expression of these factors would have to differ markedly between individuals to account for the wide variation of exon 3 splicing, and would likely affect the processing of other alternatively-spliced mRNAs.

We propose the individual-specific regulation of hGHR isoforms is the result of a cis-acting element or nucleotide sequence within the hGHR gene and that the isoforms represent different alleles. In other words, this putative cis element is a polymorphism which disrupts the normal splicing pattern and causes exclusion of exon 3 in some hGHR transcripts. The evidence for this polymorphism is twofold. First, coexpression of hGHRwt and hGHRd3 always occurred in similar quantities in our samples. Our PCR conditions were tested by mixing RNA from 100% hGHRwt and 100% hGHRd3 placentas in various proportions. The quantity of the resulting PCR products for each isoform reflected the amount of input RNA (data not shown). Second, we did not observe in any of our samples (both placenta and lymphoblastoid cell lines) a situation in which the isoform expression pattern in an individual could not have been inherited from one or the other parent.

A genetic polymorphism resulting in deletion of an entire exon from an mRNA without compromising structure or function of the resultant protein must be rare. Exon 3 encodes a segment of the extracellular domain of hGHR that is 22 amino acids in length, and its removal results in the substitution of an aspartic acid for the alanine residue at the exon 2–4 junction (2, 14). Exon 3 is not highly conserved among GHRs, and a homolog does not exist in the closely related prolactin receptor. The binding capacities of the isoforms for ligand (5, 6, 17) do not significantly differ. Finally, crystallization of the hGH·hGHR2 complex failed to indicate a critical ligand interaction for the amino acids encoded by exon 3 (18). In our studies, the incidence of individuals expressing only hGHRd3 was approximately 10%, and a significant proportion of these placentas were obtained from women who gave birth to apparently normal children. Thus, the possibility exists that exon 3 may not be critical for the proper structure and/or function of hGHR.

Alternative splicing is generally accomplished by subtle variations in the basic splicing components (19–22). The splice donor and acceptor sequences are highly conserved and, in most mRNAs, exon skipping results from suboptimal splice sites. Therefore, it was somewhat surprising that no mutations were observed in the splice sites or exons surrounding exon 3

that correlated with the hGHRd3 phenotype. Sequence alterations within introns are now being identified that have a significant impact on the splicing pathway of a number of alternatively spliced pre-mRNAs (23–25). For instance, binding of SR proteins to a purine-rich repressor element located next to the branch point in an intron in the adenovirus L1 unit effectively prevents usage of the adjacent 3′ splice site (26). It has also been shown that cis elements that are not located immediately adjacent to the splice acceptor sites can affect alternative splice site selection through alterations in secondary structure. In the hGH gene, the interaction of distal sequence elements affects the overall higher-order structure and causes alternate usage of two 3′ splice acceptor sites in exon 3 (27). Unfortunately, examination of an additional 200 nucleotides upstream of the 3′ splice site preceding exon 3 in the hGHR gene from hGHRwt and hGHRd3 individuals failed to reveal any sequence differences. Due to the size of the two introns surrounding exon 3 (>14 kb and 20 kb, respectively; ref. 14), it is impossible to predict the nature of sequence differences between hGHRwt and hGHRd3 alleles that might give rise to such a complete inclusion or exclusion of exon 3.

In summary, the isoform expression patterns in the individuals we examined were consistent with the inheritance of a codominant genetic trait. We propose the two isoforms of hGHR have arisen from a genetic polymorphism that resulted in the formation of a cis element with a profound impact on pre-mRNA splicing. The relatively high frequency of the hGHRd3 variant by itself does not suggest a clinical phenotype. However, this common polymorphism, along with other variants, could impact on a polygenic clinical phenotype. In this regard, it would be interesting to explore the potential correlation of the hGHRd3 with the expression of other GH-related traits.

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