Two Distinct Species of Human Growth Hormone-variant mRNA in the Human Placenta Predict the Expression of Novel Growth Hormone Proteins*

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We have sought direct evidence for the in vivo expression of the human growth hormone-variant (hGH-V) gene by screening a placental cDNA library with a hGH-V-specific oligonucleotide. Nine independent hGH-V cDNA clones were isolated and analyzed, and three distinct species were detected. Five of these hGH-V cDNAs represent mRNAs spliced and processed in a pattern analogous to that of the highly homologous human growth hormone and human chorionic somatomammotropin gene transcripts. Each of the remaining four hGH-V cDNAs contains an additional segment of 253 nucleotides corresponding in position and sequence to the fourth intron of the hGH-V gene. In addition, one of the mRNAs in this second group uses an alternative downstream polyadenylation site. The alternatively spliced hGH-V mRNA, which we refer to as hGH-V2 mRNA, constitutes approximately 30% of the hGH-V transcripts both in the human term placenta and in a stable mouse fibroblast line expressing the transfected hGH-V gene. The placental expression of the hGH-V gene is specific to villous tissue. The hGH-V2 mRNA is predicted to encode a protein which substitutes the 63 carboxyl-terminal amino acids of hGH-V with a new 104-residue carboxyl terminus resulting in significant divergence in their relative physical properties. The alternative splicing of the hGH-V transcripts to hGH-V and hGH-V2 mRNAs expands the potential complexity of the hGH-V gene's role in normal placental function.

The human growth hormone gene cluster contains five structural genes arranged in the same transcriptional orientation. These genes from 5' to 3' are the normal human growth hormone gene (hGH-N), a chorionic somatomammotropin-like gene (hCS-L) presumed to be nonfunctional, a functional hCS gene (hCS-A), the growth hormone variant gene (hGH-V), and a second functional hCS gene (hCS-B) (Seeburg, 1982). The hGH-N gene is expressed specifically in the pituitary, while the hCS-A and hCS-B are expressed in the syncytiotrophoblastic villous epithelium of the placenta (McWilliams and Boime, 1980). The pattern of expression of the hGH-V gene has not been characterized as completely. The cloned hGH-V gene is expressed when transfected into a variety of cell lines as a 22-kilodalton (kDa) protein (Pavlakis et al., 1981, and Footnote 2). However, evidence for its expression in vivo has been limited to the detection of a positive signal on dot blot analysis of human placental mRNA and the mRNA isolated from a single human pituitary tumor by hybridization to an hGH-V-specific oligonucleotide probe (Frankenke et al., 1987). The immunologic detection of an hGH-related, non-hCS protein in amniotic fluid and maternal blood samples, speculated to represent the hGH-V gene product, supports the possibility of hGH-V protein synthesis and secretion by the placenta (Kletzky et al., 1985; Hennen et al., 1985). In the present study we have attempted to corroborate that the hGH-V gene is expressed in the human placenta by cloning and determining the structure of hGH-V mRNA. Our results clearly demonstrate that the hGH-V gene is in fact expressed in the placenta and that it encodes two major mRNA species by alternative splicing of the fourth intron. These two mRNAs predict the expression of two distinct hGH-V proteins with significant structural divergence.

MATERIALS AND METHODS

DNA Modification and Labeling—Restriction and modification enzymes were purchased from New England Biolabs and Bethesda Research Laboratories and were used according to the manufacturers' specifications. Nick translations were carried out with the Bethesda Research Laboratories nick translation kit according to the manufacturer's instructions. Oligonucleotide fragments were end-labeled using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's instructions. Oligonucleotide fragments were end-labeled using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs) with subsequent purification by column chromatography through Sephadex G-25 spin columns (Boehringer Mannheim).

Probes and Hybridizations—The hGH-V-specific oligonucleotide 5'-TATGCCAGCTGGTACAGGCGAC-3' corresponding to codons 18 through 25 of hGH-V mRNA was prepared by the DNA Synthesis Service of the Cancer Center of the University of Pennsylvania. Human GH cDNA insert (788 base pairs) was released from phGH plasmid (Martial et al., 1979) by digestion with HindIII, resolved on a 5% acrylamide gel, and isolated by electrophoresis. The fourth intron of the hGH-V gene was isolated and subcloned by digesting the hGH-V gene (Seeburg, 1982) with BstXI, filling in the ends with the Klenow fragment of DNA polymerase and ligating this 200-base pair fragment into BamHI- and Smal-digested

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‡ The abbreviations used are: hGH, human growth hormone; hCS, human chorionic somatomammotropin; bGH, bovine growth hormone; TMAC, tetramethylammonium chloride.

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were carried out according to standard protocol (Maniatis et al., 1982), followed by prehybridization of the baked filters overnight in 6 x SSC, 50 mM sodium phosphate, pH 6.7, 5 x Denhardt’s solution containing 0.1 mg/ml sonicated denatured salmon sperm DNA, and hybridized for 24 h at 37 °C in the same buffer containing 100 mg/ml dextran sulfate and 300,000 cpm/ml of 32P-labeled oligonucleotide or insert probe. Filters were then rinsed three times at 4 °C in 6 x SSC, washed twice for 30 min each with 6 x SSC at 4 °C, rinsed with 0.1% sodium dihydrogen tartrate (TMAC) wash solution (Wood et al., 1985) at 37 °C and twice for 20 min with TMAC wash solution at 50 °C. The TMAC wash solution includes 3 M TMAC, 50 mM Tris-HCl, pH 8.0, 2 mM Na2EDTA, and 1 mg/ml sodium dodecyl sulfate.

Library Screening—A full-term human placental cDNA library constructed in lambda gt11, containing 23% recombinant phage, was the generous gift of M. Weiss (University of Pennsylvania). Details of its construction have been previously published (Henthorn et al., 1986). The library was screened (Benton and Davis, 1977) with the hGH-V-specific oligonucleotide at the empirically determined temperature and wash conditions described above. Positive plaques were purified by sequential plating and hybridization, and DNA was isolated from confluent plates lysates by standard methodology (Maniatis et al., 1982). Inserts from each of the positive phage were subcloned into M13mp18 and selected clones were sequenced by the method of Sanger et al. (1977) using both universal sequencing oligonucleotides and oligonucleotides complementary to internal regions of the cDNA (Fig. 2). Each of the reported sequences was determined from analysis of both strands or two independent analyses of a single strand, and each of the primer sites was overlapped by adjacent sequencing runs.

Expression Studies—An hGH-V expression recombinant was constructed by fusing the 5'-nontranslated region of the hGH-V gene at the BamHI site to the 5'-nontranslated region of the mouse metallothionein (MT) promoter. This chimeric gene, containing the hGH-V structural gene under the transcriptional control of the metallothionein promoter, was then inserted into a bovine papillomavirus shuttle vector (pBPV). The resulting plasmid, pBPV-MT-X-hGHV (described in detail elsewhere), was then transfected into C127 cells, a mouse mammary tumor line (Law et al., 1981; American Type Culture Collection line CRL-1616) by Ca3(PO4)2 coprecipitation followed by shock treatment with glycerol at 20% final concentration for 1 min (Howley et al., 1983). Individual transformed foci were selected and expanded. Cells were maintained at 37 °C with 5% CO2 in Eagle’s modified minimal essential media containing 8% fetal bovine sera. Once confluent, the cell lines were tested for hGH-V expression. The cells were washed twice with phosphate-buffered saline and plated in serum-free medium for 24 h, after which the medium was assayed for hGH-V protein by enzyme-linked immunosorbent assay (Engvall, 1980) or Western analysis using a polyclonal anti-hGH antibody (Liebhaber et al., 1986).

RNA Isolation and Analysis—RNA was prepared from tissue culture cells by the method of Strohman et al. (1977). RNA was isolated from a fresh human term placenta after dissection into amnion, chorion, villous, and decidual layers (Chirgwin et al., 1979). Polyadenylated RNA was isolated using Hybond-mAP (Amersham Corp.) following the recommendations of the supplier. Total and/or polyadenylated RNA samples were denatured in 6.6% formaldehyde at 65 °C for 15 min and either applied directly to nitrocellulose by dot blotting or electrophoresed through 1.8% agarose, 6.6% formaldehyde submerged slab gels (Lehrach et al., 1977), and transferred to nitrocellulose filters (Thomas, 1980). The filters were hybridized with either oligonucleotide or insert probes as described above.

RESULTS

A human term placental cDNA library was screened for hGH-V cDNAs. To specifically detect hGH-V sequences as opposed to those of the closely related hCS or hGH-N genes, the library was screened with a 22-base synthetic oligonucleotide which differed from the homologous region of hCS by 7 bases and from the homologous region of hGH-N by 5 bases. After determining conditions at which this probe was specific for the hGH-V mRNA (Fig. 4A), 2 x 105 individual plaques representing approximately 4 x 104 individual recombinants of the placental library were screened. 22 positives were identified and confirmed by secondary plating. Nine of these positive phage were chosen at random. These were then plaque-purified and analyzed for insert size. The inserts ranged from approximately 700 to 1000 base pairs, compared to the near full-length 833-base pair hGH cDNA insert analyzed in parallel (Fig. 1A).

Clone V7 (see Fig. 1A) which contained the largest insert was selected for initial DNA sequence analysis. The sequencing approach is shown in Fig. 2 and the primary structure of this hGH-V cDNA is shown in Fig. 3. When compared with the hGH-V gene structure previously reported, the sequence of this cDNA begins at residue -20 of the signal peptide, extends through the 5 exons, and ends with a poly(A) tail at the expected position. In addition there is an internal 253-nucleotide segment which correlates in position and sequence to intron 4 of the hGH-V gene. The sequence of the intron 4 region of this cDNA is identical to that reported for the hGH-V gene, with the exception of a single guanine and a double cytosine insertion in the region immediately 3' to the start of intron 4 (indicated by plus signs in Fig. 3).

To determine whether the structure of V7 cDNA represented a common or rare alternative splicing event, the nine cDNA clones were rehybridized with the labeled insert of an intron 4 subclone. As seen in Fig. 1B, four of the nine hGH-V cDNA clones contain intron 4 sequences, suggesting that a significant proportion of placental hGH-V mRNAs is generated by alternative splicing of intron 4. To confirm the structure.
The goal of this study was to determine whether the hGH-V gene was expressed in vivo and, if so, to isolate the hGH-V mRNA and define its structure. Detailed analysis of nine clones selected with a specific oligonucleotide resulted in the definition of three separate hGH-V mRNA structures, two major and one minor. The first of the two major mRNAs is generated by the splicing pattern originally predicted for the hGH-V transcript on the basis of the structure of the closely related hGH-N and hCS mRNAs. The second major mRNA species is generated by an alternative splicing pattern in which the last intron (intron 4) of the hGH-V transcript is retained in the mRNA. A third structural variation detected in a single hGH-V2 cDNA contains a 4-base extension at the poly(A) addition site. This last variant structure, although of no obvious functional significance, may reflect the utilization of the AAUAAA hexamer as a minor poly(A) additional signal. This signal, which has been previously recognized as a functional hexamer for polyadenylation (Birnstiel et al., 1985), begins 5 bases downstream of the predominant polyadenylation signal AAUAAA. Although no direct attempts at quantitation are reported here, it can be roughly estimated from the representation in the cDNA library that hGH-V and hGH-V2 mRNAs together constitute approximately 0.05% of total placental mRNA. These studies therefore confirm that the hGH-V gene is expressed in vivo and that there is an unexpected heterogeneity in the structure of its encoded mRNAs.

Our analysis of mRNA isolated from the major anatomic regions of the placenta was undertaken to sublocalize the expression of the hGH-V gene. The results demonstrate that the expression of the hGH-V gene is limited to the villous tissue. This is consistent with the immunologic sublocalization of the closely related hCS gene product to syncytiotrophoblasts within the villous epithelium (McWilliams and Boime, 1980). From the present data it cannot be determined whether the same syncytiotrophoblast cells express both hCS and hGH-V. This possibility may be important since these two homologous genes may share regulatory sequences that serve to distinguish their pattern of expression from the pituitary-specific hGH-N gene. For this reason it will also be important to determine whether the recent report of very low levels of hGH-V mRNA in a single human pituitary tumor (Frankenne et al., 1987) was an aberration of neoplastic growth or whether the hGH-V gene is normally expressed (even at low levels) in the pituitary. A comparison of the expression of the three highly homologous GH genes, hGH-N, hGH-V and hCS, should provide an interesting system for the study of transcriptional signals controlling tissue-specific gene expression and coordinate gene regulation.

The present demonstration of alternative splicing of hGH-V gene transcripts can be compared to a recent study which detected intron 4 sequences in a fraction of bovine GH (bGH) mRNA (Hampson and Rottman, 1987). While it is likely that these bGH mRNAs represent alternatively spliced transcripts of a single bGH gene, their structure has not yet been firmly established by sequence analysis. Since Southern blot analysis suggests there may be two GH genes in the bovine genome (Gordon et al., 1983), the possibility remains that the intron 4 containing bGH mRNAs could represent the transcripts of a second bGH or bGH-related gene. Despite these reservations, the presence of intron 4 in a mature bGH or bGH-related mRNA is potentially significant. Although the proportion of bGH transcripts containing intron 4 (0.1%) is lower than that of hGH-V (30%), the higher total concentration of bGH mRNAs in the pituitary (approximately 10%) than hGH-V mRNAs in placenta (approximately 0.05%) results in the expression of comparable levels (approximately 0.01%) of intron D containing mRNAs in the respective tissues. In addition, introns 4 of the bGH and hGH (and hGH-V) genes possess a remarkable degree of nucleotide identity, higher in

**DISCUSSION**

![Diagram of hGH-V mRNA structures](image)

The three additional intron 4 containing cDNA clones, each was sequenced, either partially or completely (Fig. 2). The only difference noted in their sequences was a 4-nucleotide 3' extension in clone V9.

To characterize the remaining cDNAs lacking intron 4, two of them (V1 and V8) were fully sequenced. Clone V1 extended from codon -22 in the signal peptide through to the poly(A) tail. Its structure includes only the 5 previously reported exons of the hGH-V gene. The second clone, V5, began at codon 5 and was otherwise identical in sequence to clone V1.

To sublocalize hGH-V gene expression in the placenta and to characterize further the variable splicing of its transcripts, we analyzed mRNA from a number of tissues in the human term placenta and from a stable mouse fibroblast cell line expressing a transfected hGH-V gene. Northern analyses of mRNA from the four placental tissues, amnion, chorion, villi, and decidua, were hybridized with the subcloned hGH-V intron 4 fragment. The results of this study, shown in Fig. 4B, demonstrated a single mRNA band of 1250 nucleotides, the expected size of hGH-V2 mRNA, which was limited to the villous RNA. A dot blot analysis of mRNA from each of these tissues probed with the hGH-V-specific oligonucleotide confirmed that all hGH-V gene transcripts are limited to the villous tissue (data not shown). The question of whether the variable splicing was specific to the placenta was approached by Northern analysis of mRNA from the hGH-V-transfected cell line. This study demonstrates two mRNA species of approximately 900 and 1250 nucleotides (Fig. 4C, lane 1). These sizes are consistent with the expected size of the hGH-V and hGH-V2 mRNAs, respectively. The relative concentrations of the two species as determined by densitometric analysis is 2:1 (hGH-V:hGH-V2). This ratio is consistent with the ratio of the hGH-V and hGH-V2 clones isolated from the placental library (5:4). Rehybridization of this filter with the intron 4 probe confirmed that the 1250-nucleotide mRNA was hGH-V2 (Fig. 4C, lane 2). We conclude that hGH-V and hGH-V2 mRNAs are major alternative splicing products of the single hGH-V gene and that this alternative splicing pattern is not tissue-specific.
Fig. 3. The sequences of the hGH-V and hGH-V2 cDNAs. The numbering begins with the first base of the mRNA as defined from the genomic structure (Seeburg, 1982). Where the numbering differs between the hGH-V and the hGH-V2 mRNAs, the hGH-V2 sequence is shown above bases 535, 540, and 541 above. The two different 3’ ends of the hGH-V2 mRNA are indicated with the less prevalent form in parentheses. The plus signs (+) above bases 535, 540, and 541 in the hGH-V2 sequence indicate the positions of the bases in our sequence which are not present in a previously reported intron 4 sequence (Seeburg, 1982). The dot above base 75 represents the furthest 5’ extent of our hGH-V mRNA is unclear. The relative concentrations of the two major hGH-V mRNAs as estimated by the structural analyses of the nine cDNA clones (Fig. 1) and more quantitatively by Northern analysis (Fig. 4), demonstrate that hGH-V2 constitutes one-third or more of total hGH-V mRNA. The functional significance of the alternative splicing of this exon to intron 4 is discussed below.

The functional significance of the alternative splicing of the hGH-V mRNA is unclear. The relative concentrations of the two major hGH-V mRNAs as estimated by the structural analyses of the nine cDNA clones (Fig. 1), and more quantitatively by Northern analysis (Fig. 4), demonstrate that hGH-V2 constitutes one-third or more of total hGH-V mRNA. The
The specificity of the hGH-V oligonucleotide probe is demonstrated by dot blot analysis of recombinant plasmids containing the hGH-N (N), the hGH-V (V), or the hCS (CS) genes. Each of these three plasmids was linearized, applied to nitrocellulose paper, and hybridized with either a hGH-N cDNA probe (lane 1) or with an oligonucleotide specific for the hGH-V gene transcripts (lane 2). A poly(A+) mRNA from placental amniotic membranes (Am), chorionic membranes (Ch), villous (Vi) or decidual tissue (De) was fractionated on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose paper, and hybridized with an hGH-V intron probe. An ethidium bromide stain of the gel prior to transfer demonstrates residual 28 S ribosomal RNA in each of the lanes. The position of the 18S and 28S rRNAs is marked to the left of the autoradiograph. The size of the hybridizing hGH-V2 mRNA band shown on the right of the autoradiograph was determined by comparison with RNA size markers on the same gel (not shown). C. RNA from a stable mouse fibroblast cell line containing the transfected hGH-V gene was analyzed as in B and hybridized with the oligonucleotide probe specific for hGH-V gene transcripts (see A) (lane 1) or with the hGH-V gene intron 4 probe (lane 2). nt, nucleotide.

The protein products of the two hGH-V mRNAs are predicted to differ significantly in their structures. Excluding post-translational modifications other than signal peptide cleavage, the hGH-V2 mRNA would encode a mature 26-kDa protein with a pI of 6.5, whereas hGH-V mRNA would encode a mature 22-kDa protein with a pI of 9.3. The hGH-V2 protein would differ from hGH-V and all of the GH-related proteins thus far examined in the locations of its intramolecular disulfide bonds. Additionally, it would have lost the single predicted N-linked glycosylation site at Asn-140, unique to hGH-V among the GH-related genes. A particularly interesting difference between the structures of the hGH-V and the predicted hGH-V2 proteins was detected in an analysis of their hydrophobicity profiles. The predicted hGH-V2 protein contains in its carboxyl terminus a hydrophobic region of approximately 12 amino acids bracketed by two extended hydrophilic regions (Fig. 5). This configuration of residues is consistent with a membrane-spanning region (Kyte and Doolittle, 1982) and suggests the possibility that the hGH-V2 protein may be an integral membrane protein. Recent reports of circulating GH receptors (Herington, 1986; Leung et al., 1987) and their ontogeny during fetal life (Daughaday et al., 1987) suggest a potential ligand for a membrane-bound growth hormone. These findings plus the presence of GH-releasing factor in placentae (Baird et al., 1985) and membrane-bound somatomedin receptors and somatomedin binding protein in amniotic fluid and in the placenta suggest the presence of a complete GH regulatory system (Pooke et al., 1984). If a membrane-bound hGH-V2 protein can be demonstrated, the potential physiologic function and mechanism of action of hGH-V2 might differ significantly from the spectrum of functions and mechanisms presently considered for hGH-N and its related proteins.

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