Developmental Control and Alternative Splicing of the Placentally Expressed Transcripts from the Human Growth Hormone Gene Cluster*

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Four of the five genes in the human growth hormone gene cluster are expressed in the villous layer of the placenta. We report that the expression of these genes, hCS-A, hCS-B, hCS-L, and hGH-V, are coordinately induced during fetal development, increasing between 12 and 20 weeks of gestation and then plateauing through term. Within the context of this coordinate activation, these genes are expressed at widely different levels and are alternatively spliced in different patterns. There is a developmentally regulated switch in the relative expression of the two chorionic somatomammotropin genes, hCS-A and hCS-B. Starting from approximately equal levels at 8 weeks of gestation, hCS-A is expressed 5-fold more abundantly than hCS-B by term. The proportion of alternatively spliced hGH-V transcripts that retain intron 4 is also developmentally regulated, increasing 3-fold during gestation to 15% at term. A small percentage of hCS transcripts stably retain intron 4 through gestation, the majority derived from the hCS-A gene. hCS-L transcripts undergo two distinct, developmentally stable, splicing pathways between exons 2 and 3. These result from the absence of the normal splice-donor site in intron 2 and the activation of two cryptic splice-acceptor sites. Despite high levels of sequence identity, the four placentally expressed genes in the growth hormone cluster generate a complex set of mRNAs based on alternative splicing and developmental regulation during gestation.

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The human growth hormone (hGH) gene cluster, located on chromosome 17q22–24 (1), contains five structural genes that have evolved through a series of duplication events (2). The genes are all positioned in the same transcriptional orientation and display more than 90% nucleotide sequence identity in their coding and immediate flanking regions (3, 4). Despite this structural similarity, the genes are expressed at different levels and in different tissues. The normal growth hormone gene, hGH-N, is expressed by somatotropes in the anterior pituitary. The other four genes are expressed by syncytiotrophoblasts in the villous layer of the placenta (4). Two chorionic somatomammotropin genes (hCS-A and hCS-B) encode identical hCS proteins that can be specifically identified in syncytiotrophoblasts using immunofluorescent staining as early as 12–18 days postconception (5). During the second and third trimesters of pregnancy, concentrations of hCS rise in maternal sera to a range of 3–25 µg/ml (6). Growth hormone-variant (hGH-V) gene transcripts are also found in the placental villi (7, 8) but at levels at least 2 orders of magnitude lower than hCS-A and hCS-B (4). The hGH-V gene encodes a secreted protein (9, 10) that increases in parallel with hCS in maternal sera (11). The final member of the cluster, the chorionic somatomammotropin-like (hCS-L) gene, had been regarded as a nonexpressed pseudogene (12), although identification of hCS-L cDNA clones in a term placental library (4) suggests that it is transcriptionally active.

Genes of the GH cluster differ not only in tissue distribution and levels of expression but also in patterns of splice-site selection. The predominant splicing pattern shared by hGH-N, hGH-V, hCS-A, and hCS-B transcripts involves the ligation of five common exons. These mRNAs encode secreted proteins of 191 amino acids which display 85% or greater amino acid sequence identity. Approximately 10% of pituitary hGH-N transcripts utilize an alternate splice-acceptor site 45 bases within exon 3 (9, 13, 14) resulting in a 15-amino acid internal deletion in the expressed hormone. This 20-kDa hGH isoform has a selective loss of insulin-like bioactivity compared with the normal 22-kDa hGH (15). Surprisingly, the hGH-V transcripts, sharing greater than 95% nucleotide identity with hGH-N, do not utilize the alternative splice-acceptor site in exon 3 (9, 14). Instead they undergo an alternative splicing pathway in which intron 4 is retained in the processed

1 The abbreviations used are: hGH, human growth hormone; hCS, human chorionic somatomammotropin; hCS-L, human chorionic somatomammotropin-like; hGH-N, normal human pituitary growth hormone; hGH-V, human growth hormone-variant (alternatively known as placental growth hormone); hGH-V2, hGH-V spliced to retain intron 4; hCS-A2/hCS-B2, hCS-A/hCS-B spliced to retain intron 4; hCGB, human chorionic gonadotropin β-subunit; MOPS, 3-(N-morpholino)propanesulfonic acid; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s).
mRNA (7). The hGH-V transcripts that retain intron 4 predict the expression of a 25-kDa protein isoform, hGH-V2, containing a unique 104-amino acid carboxyl terminus (7). The reported splicing between hCS-L exons 2 and 3 differs from the others. The hCS-L intron 2 splice-donor site has been lost because of a single-base substitution resulting in activation of cryptic splice-donor and splice-acceptor sites (4). Although there is no evidence for an hCS-L protein product, these hCS-L splicing pathways do not change the reading frame of the processed mRNA, and a minimally altered hCS isoform could theoretically be synthesized.

In the present report, we compare the placental expression of hCS-A, hCS-B, hCS-L, and hGH-V throughout gestation by measurement of steady-state mRNA levels and analysis of mRNA structure. We conclude that the genes of the hGH cluster have a developmentally coordinated pattern of expression but are differentially regulated both transcriptionally and by alternative splice-site selection.

MATERIALS AND METHODS

Placental Tissue—Human placentas were obtained from a nonsmoking population of women at various stages of gestation, 5 weeks through term, delivering on the obstetrical service at the Hospital of the University of Pennsylvania. All tissues were obtained following examination by the pathology department under a protocol that was approved by the University of Pennsylvania Committee on Studies Involving Human Beings. The villous layer was dissected from each intact placenta, frozen in liquid nitrogen, and stored at -70 °C until the time of RNA isolation.

RNA Isolation—Total cellular RNA was isolated from placental villous tissues by lysis in 5 M guanidine thiocyanate, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.75 M 2-mercaptoethanol followed by centrifugation through a 5 M CsCl cushion (16). The pellet RNA was resuspended in 1 M Tris-HCl, pH 7.4, and 1 mM EDTA containing 0.1% (w/v) N-lauroylsarcosine and 5% (v/v) glycerol, and ethanol precipitated. Final RNA pellets were dissolved in sterile water and stored at -70 °C.

Northern Blot Analysis—Total villous RNA, 10 μg sample, was electrophoretically separated through 1.5% agarose, 6.5% formaldehyde gels, submerged in buffer (pH 7.0) containing 40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA. Prior to loading, each sample was denatured by heating to 55 °C for 15 min in buffer containing 50% deionized formamide, 40 mM MOPS, and 6.5% formamide. The separated RNAs were then transferred to GeneScreen Plus nylon membranes (Du Pont-New England Nuclear) using the capillary blotting technique with 10 × SSC (17). Prehybridization and hybridization were carried out at 42 °C in buffer composed of 5 X SSPE, 5 X Denhardt’s solution, 10% (w/v) dextran sulfate, and 1% (w/v) sodium dodecyl sulfate, 60% deionized formamide, and 100 μM α-labeled salmon sperm DNA. High stringency wash conditions followed the recommendations of the membrane’s manufacturer.

To isolate an hCS cDNA, a term placental cDNA library (7) was screened with an hGH-N CDNA probe. The selected clone contained an 840-bp EcoRI insert that was sequenced (18) from each EcoRI site. The 5′ border coincided with codon 9 of hCS mRNA, and sequencing confirmed the presence of the normal exon 2 to exon 3 splicing junction, indicating that the clone was not hCS-L. The sequence was identical to that of hCS-A (4) cDNA with the exception of three single-base differences in the 3′-nontranslated region. The coding region was followed by a 77-base poly(A) tail. Additional hybridization probes included human chorionic gonadotropin-β (hCG-β) cDNA (19) and an 18 S ribosomal genomic probe (20), a kind gift of J. Sylvester (Hahnemann University). All probes were released from their plasmid vectors, gel purified, and 32P labeled using a random-primed DNA labeling kit (Boehringer Mannheim).

Reverse Transcription and Polymerase Chain Reaction (RT/PCR—CDBAs were synthesized from 2 μg of total villous RNA using 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) under conditions described previously (8). Gene-specific oligonucleotide primers (Table 1) were synthesized (Pharmacia LKB Biotechnology Inc., Piscataway, NJ; Applied Biosystems Inc., Foster City, CA) to prime both the reverse transcription reaction and the polymerase chain reaction amplification. The amplifications utilized the same antisense primers as the RT reactions plus a gene-specific 32P end-labeled 5′ sense primer. The two reactions were carried out sequentially. Following RT, the synthesized cDNAs were extracted with phenol/chloroform and ethanol precipitated, and then amplified using Taq polymerase (New England Biolabs, Inc., Beverly, MA) in a thermocycler (Perkin-Elmer Cetus Instruments). A unique 104-amino acid carboxyl terminus (4). Further restriction mapping of this clone with the hCS-B genomic clone was screened by hybridization with a unique XhoI site that is extracted with the hCS-A and hCS-B genes of the cluster (4). Further restriction mapping of this clone with PstI detected a single internal site, classifying the gene as hCS-B (4). A BamHI-EcoRI fragment of the hCS-B gene was subcloned into the unique XhoI site of the bovine papillomavirus, mouse metallothionein shuttle vector, pBPV-MTX (24), previously used to express other members of this gene family (9). The hCS-B fragment begins 1 base 3′ of the cap addition site and extends approximately 820 bp 3′ of the polyadenylation signal. With hCS-B oriented to be under the transcriptional control of the constitutive mouse metallothionein promoter was selected based on its SstI restriction map. This clone, pBPV-MTX-hCS-B, was then cotransfected with pHSVneo (25) at a 1:10 molar ratio into the mouse mammary tumor cell line C127 by calcium phosphate coprecipitation (26). Transfected cells were selected by growth in G-418 (27), individual clones were isolated with cloning rings, and each clone was expanded and tested for hCS-B expression by both Western blotting and enzyme-linked immunosorbent assay using polyclonal antisera to hCS (gift of the National Hormone and Pituitary Program of the NIDDK). One expressing cell line, CM-hCS-B, was used in these studies.

RESULTS

Expression of the hGH Gene Cluster in the Developing Placenta—RNA samples were obtained from the villous layer of placentas ranging in gestational age from 8 to 39 weeks. These samples were initially analyzed by Northern blotting. Identical filters prepared as parallel panels on the same gel were hybridized with the various probes (Fig. 1A). Hybridization with the hCG-β cDNA probe confirmed the gestational ages of the placentas. The levels of hCG-β mRNA/μg of total villous RNA were highest in the 8- and 10-week samples and then declined sharply. The levels of hCS mRNA, in contrast, showed a reciprocal pattern of developmental control. Denitometric quantification of these data, normalized for hybridization to the 18 S ribosomal probe, demonstrated that the concentration of hCS mRNA increases sharply between 12 and 20 weeks of gestation, then plateaus and remains rela-
blots were hybridized to 32P-labeled DNA probes corresponding to cally and transferred to a nylon membrane in triplicate. Individual is the sum of both. Furthermore, it is technically difficult to detect the low levels of hGH-V and hCS-L mRNAs with transcript-specific oligo-nucleotide primers. To achieve the desired level of mRNA sequence identity among the genes in the hGH cluster pre- mRNAs from 1.5:1.0 during the first trimester to approximately 5.0:1.0 at term. This developmental shift in gene expression was confirmed in four separate experiments, with duplicate amplifications using two independent sets of oligo-nucleotide primers.

Developmental Regulation of hGH-V2 Levels—Superimposed on the developmental profile of hGH-V expression is a proportional increase in the amount of the alternatively spliced, intron 4-containing, hGH-V2 mRNA. This was initially inferred by analysis of three timed placental samples (8). Here this is confirmed using a more complete series of gestationally timed samples and an RT/PCR scheme that coamplifies hGH-V and hGH-V2 in a single reaction tube. To maximize the accuracy of comparison between hGH-V and hGH-V2 mRNAs, the amounts of placental RNA assayed were adjusted to give relatively equal hGH-V signals from time point to time point by using more RNA from the samples taken earlier in gestation. The quantified data from the gel analysis (Fig. 4A) and nine additional timed placental RNA samples are presented in Fig. 4B. Combined, the data show a 3-fold rise (5% in the first trimester to 15% at term) in the relative amounts of hGH-V2 steady-state mRNA.

Alternative Splicing of hCS-A and hCS-B—Using hCS-A and hCS-B primers encompassing exons 2–5 in RT/PCR studies, low levels of a fragment about 250 bp larger than expected were noted (data not shown). The size of this band suggested that it might represent a transcript retaining intron 4, analogous to hGH-V2 mRNA (7, 9). To test this, RT/PCR was primed using an antisense oligonucleotide complementary to a region of hCS intron 4 identical in hCS-A and hCS-B genes but divergent in hGH-V (Fig. 5 and Table I, hCS-A2 and hCS-B2 primers). This set of primers produced a single 420-bp RT/PCR fragment in placental villous RNA, the size predicted for an intron 4-retaining hCS mRNA. To determine whether the fragment was derived from an hCS mRNA species or was generated by cross-amplification of placental hGH-V2, a mouse fibroblast cell line (C127) was transfected with the hCS-B gene, and a clonal line stably expressing hCS-B was selected and expanded. RNA from this line, CM-hCS-B, was subjected to the same RT/PCR assay. The intron 4-containing mRNA species was detected in the hCS-B cell line, with no significant cross-amplification of hGH-V2 in the stably transfected hGH-V cell line, CM-hGH-V (9), known to express 25% of its transcripts in the hGH-V2 form (Fig. 5B).

Analytical restriction digestions of the 420-bp placental RT/PCR fragment confirmed its identity as an hCS transcript retaining intron 4. Exons 4 of hCS-A and hCS-B contain unique EcoNI sites, whereas intron 4 of hGH-V contains a unique BamHI site (Fig. 5A). EcoNI digestion had no effect on an amplified hGH-V2 fragment but shortened both the placental hCS and CM-hCS-B cell line fragments from 420 to 316 bp. In contrast, BamHI reduced the hGH-V2 RT/PCR fragment from 392 to 344 bp without altering hCS fragment sizes (Fig. 5C). These digestions confirm that placental hCS
transcripts contain an alternatively spliced, intron 4-retaining product. To determine the relative intron 4 retention in hCS-A and hCS-B genes, a unique BstEII site in hCS-A2 which shortens the fragment from 420 to 372 bp was utilized (Fig. 5D). As an internal control for complete BstEII digestion, nonlabeled human 2-globin cDNA containing a single BstEII site was added to each sample tube and analyzed in parallel by ethidium bromide staining (data not shown). BstEII digestion had no effect on the amplified fragment in the CM-hCS-B cell line. In contrast, the majority of the placental fragments were digested with BstEII and therefore represent hCS-A transcripts (Fig. 5D). By analogy with hGH-V2, the hCS intron 4-retaining transcripts are referred to as either hCS-A2 or hCS-B2. Analyses of the timed series of placental samples using RT/PCR followed by BstEII digestion demonstrate that the ratio of hCS-A2 to hCS-B2 increases from 2.6 to 12.0 during gestation (data not shown). This 4.6-fold rise closely parallels the 5.0-fold increase observed in the hCS-A/hCS-B ratio (Fig. 3). This parallel pattern suggests developmental stability in the rate of intron 4 retention. The higher ratio of hCS-A2/hCS-B2 compared with hCS-A/hCS-B at each gestational time point suggests that intron 4 is retained twice as efficiently by hCS-A.

Two previous sequencing reports disagree on the sequence of intron 4 of hCS-A at 5 positions (4, 28). One sequence would predict an open reading frame extending through intron 4 of hCS-A2 mRNA (28), whereas the other (4) would predict a termination codon 62 amino acids into the intron. To resolve this ambiguity, an hCS-A genomic clone and intron 4 PCR products from two term placental samples were sequenced, five hCS-A alleles in total. The sequences determined were identical with the exception of a previously unreported A/C polymorphism at position 1241 in one of the placental samples. Our sequences differ from that of Chen et al. (4) at positions 1271, 1300, and 1324 and from Selby et al. (28) at positions 1128 and 1245 (Fig. 6). The revised sequence predicts an open reading frame in the intron 4 region of hCS-A2 mRNA. This open reading frame enters exon 5 with a 1-base frame shift compared with the reading frame in hCS-A mRNA, resulting in the prediction of an hCS-A2 protein of 230 amino acids. This protein would be the same length and share 80% amino acid identity with the protein predicted for hGH-V2.

**Alternative RNA Splicing of hCS-L**—Very little information is available on the expression of the hCS-L gene. Published data are limited to a description of 10 hCS-L cDNA clones, 9 of which were considered to be incompletely processed hRNAs (4). To detect hCS-L mRNA in the placenta, we primed cDNA synthesis with an antisense primer that hybridizes to sequences encoded in exon 5. The cDNA was amplified between this primer and one of two sense primers (Table I). The first sense primer includes the 19-base, 3' extension of which would be the same length and share 80% amino acid identity with the protein predicted for hGH-V2.

**Table I**
The 5' and 3' primers used in the RT/PCR reactions

| Primer | Length | Position | Orientation | Sequence (5' to 3') |
|--------|--------|----------|-------------|---------------------|
| hGH-V  | 5'     | Ex 2     | +           | GCCGCTCTACACGCTGCCATA |
|        | 3'     | Ex 4/5   | –           | ATCTTCCAGCCCTCAGATCA |
| hGH-V2 | 5'     | Ex 2     | +           | TGGCTGACTACCACTGGATCA |
|        | 3'     | In 4     | –           | TCTTCTCCACGGCTCCACT |
| hCS-L  | 5'     | Ex 2     | +           | AGGATTTAAGTCTTGGGGAAATG |
|        | 3'     | Ex 5     | +           | GAGGCTCCTCCCTGCTGCT |
| hCS-A  | 5'     | Ex 5     | +           | AAACACTACGGCTCTCTT |
|        | 3'     | Ex 5     | +           | GCCGCGAGGAACGACAT |
| hCS-A2 | 5'     | Ex 2     | +           | CTGGTTCAGCAGCTAGCTT |
|        | 3'     | In 4     | –           | TCTTCTCCACGGCTCCCT |
| hCS-B  | 5'     | Ex 5     | +           | AAGACTAGGGCTCTCTT |
|        | 3'     | Ex 5     | +           | GCCGCGAGGAACGACAT |
| hCS-B2 | 5'     | Ex 2     | +           | CTGTTCACAGCTAGCTT |
|        | 3'     | In 4     | –           | TCTTCTCCACGGCTCCCT |
| hGH-N  | 5'     | Ex 3     | +           | CTAATACCGCAAGACAGA |
|        | 3'     | Ex 5     | –           | GGCGCTCGCATCTCCAG |
| β Actin| 5'     | Ex 3     | +           | CTCAAACTACGGTTCGTTG |
|        | 3'     | Ex 4     | –           | CAGTCTCCAGCAGCGAT |

*The A in the AUG initiation codon is defined as +1.*

*Strand orientation: sense (+), antisense (−).*
analyzed during the logarithmic phase of amplification. Total RNA and hCS-B mRNAs (Fig. 2) were determined by direct Phosphor-analysis in parallel to serve as a negative control. Amplification of placental villous RNA samples. β-actin mRNA is present at very low levels in reticulocyte RNA, composed primarily of globin transcripts. The smaller transcript (hCS-L) contained about 88% of the total counts compared with 12% for A, hCS-B, and hGH-V. The smaller transcript (hCS-L) displayed a developmental profile roughly comparable to hCS-A, hCS-B, and hGH-V. The smaller transcript (hCS-L') contained 85% of the total counts compared with 15% for hCS-A and hCS-B.

The size of hCS-L was that expected from the described exon 2 to exon 3 splice (4). To confirm this structure and to determine the structure of hCS-L', both amplified fragments were gel purified and directly sequenced. The DNA sequence of hCS-L corresponded exactly to that expected (4). The hCS-L' DNA sequence contained the same 19-base 3' extension of exon 2 as hCS-L mRNA but utilized a distinct, cryptic splice-acceptor within intron 2. This novel splice-acceptor site is located 4 bp 3' from the position of the normal splice-acceptor site utilized by the other genes in the cluster (Fig. 7). Both the hCS-L and hCS-L' mRNAs maintain the same reading frame and predict proteins after signal peptide cleavage of 174 and 197 amino acids, respectively. Excluding the deleted portions of exon 3, the predicted proteins for hCS-L and hCS-L' share roughly 91% amino acid identity with hCS.

**DISCUSSION**

Although the five genes of the GH cluster share a high degree of structural similarity, the expression of each is uniquely controlled by variations in the timing and level of gene expression and the utilization of divergent splicing pathways. Despite sequence similarity and tight linkage, members of the cluster also demonstrate mutually exclusive tissue specificities. In this report, mRNA steady-state levels and mRNA structures of the placently expressed members of this cluster are compared throughout gestation.

The parallel rise in circulating concentrations of hCS and hGH-V protein in maternal sera during gestation is well established (11) and may reflect placental growth (29) or a transcriptional increase in gene expression (30, 31). In the present study a 5–10-fold increase in hCS-B, hCS-L, and hGH-V mRNA levels per unit of villous tissue was detected between 9 and 39 weeks of gestation (Figs. 1 and 2). Over the same period hCS-A mRNA levels increase 30-fold. Since the relative increase in mRNA concentrations in the placental villi are at least 10-fold below the increases in circulating levels of hCS and hGH-V hormones (29, 11), we conclude that the developmental profiles of these hormones in maternal sera reflect increases in both placental mass and transcriptional activity. Although these findings suggest parallel increases in the transcriptional activation of these genes, other mechanisms affecting steady-state mRNA concentrations such as transcript processing, mRNA transport, and mRNA stability have not been excluded.

The differential regulation of hCS-A and hCS-B gene expression during development was unexpected (Figs. 2 and 3). A ratio of hCS-A to hCS-B mRNA at term of 6.0 had been reported previously (4). However, we noted that the ratio in the first trimester is only 1.5, gradually shifting to 5.0 by term. This shift in relative expression occurs in parallel with induction of high levels of expression. Since hCS-A and hCS-B both encode identical proteins, the physiologic necessity for such a switch is unclear. The molecular mechanism regulating the shift is also obscure. The hCS-A and/or hCS-B genes are known to utilize two transcriptional start sites, although the relative utilization by each gene has not been determined. The major site (95%) lies 30 bases downstream from the TATAAA; the minor site (5%) lies 30 bases downstream from CATAAAA, which itself is 55 bases 5' to the TATAAA (28). The relative usage of these promoter sites by each gene throughout gestation might contribute to the discrepant levels of expression. Transcriptional regulation associated with the GHF-1/Pit-1 sites of the two genes have not been compared in parallel experiments but appear from sequence analysis
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Fig. 4. Increase in the relative amount of hGH-V2 mRNA compared with normally spliced hGH-V mRNA in placental villous tissue throughout gestation. A, total RNA prepared from human placental villous samples, human reticulocytes, and cloned hGH-V and hGH-V2 cDNAs was analyzed by a three-oligonucleotide RT/PCR assay that complements hGH-V and hGH-V2 mRNAs in parallel. This is shown schematically. The assay uses a common 5’ end-labeled 5’ oligonucleotide primer and two transcript-specific 3’ oligonucleotide primers. The two cDNA controls amplify only bands of the predicted size. Reticulocyte RNA is negative for both transcripts. To permit a direct visual comparison of the relative levels of hGH-V and hGH-V2 transcripts, sample volumes were normalized to allow for approximately equal amounts of hGH-V mRNA. B, relative levels of hGH-V2 splicing during gestation. The signals in a series of experiments, represented by panel A, were quantified from autoradiographs by densitometric scanning and plotted as a function of gestational age, correlation coefficient \( r = 0.85 \). To overcome inter-experimental variations, data were normalized to the average first trimester value in each assay.

and independent functional studies to be similar (32, 33). The sequences of other potential cis-acting regulatory elements (34-37) are identical for the two hCS genes. One of these, an enhancer sequence identified 3’ of the hCS-B gene, was found to be inactive in the analogous region 3’ of the hCS-A gene (35). However, this would predict lower expression of hCS-A than hCS-B, not the pattern observed. Finally, the relative expression of the two hCS genes might be dictated by their relative positions within the activated GH gene cluster.

Transcriptional regulation of the two human α-globin genes is similar to that of hCS-A and hCS-B. Like the hCS genes, the two α-globin genes have greater than 95% structural identity and encode identical proteins (38, 39). Like the hCS genes, the relative expression of the two α-globin genes is equal during early embryonic development but diverges as development proceeds with the more 5’ gene predominating (40). By adulthood α2-globin gene expression exceeds that of α1-globin by 2.6-fold (41, 42). It has been possible to recapitulate the differential developmental control of the two α-globin genes in transgenic mice (43), and this transgenic model should make it possible to delineate the relative contributions of cluster organization versus cis-acting elements to differential regulation. Based on the parallels between these two systems, similar approaches should be possible for the study of the hCS loci.

Alternative splicing of the ghGH-V transcript was initially detected by isolation of hGH-V cDNAs from a placental library; four out of nine hGH-V cDNA clones were found to contain intron 4 and were designated hGH V2 (7). Analysis of placental RNAs at 9, 25, and 40 weeks of gestation suggested an increase in the relative percentage of hGH-V2 mRNA as gestation proceeded (8). The additional 17 timed placental samples used in the current study confirm this finding (Fig. 4). The relative amount of hGH-V2 increases from 5-7% of the total hGH-V mRNAs in the first trimester to approximately 15-20% at term. Such developmental regulation of alternative splicing has been well documented in a number of systems (44, 45). From our current data, the relative increase in hGH-V2 transcript level during gestation may reflect developmental control over splice site selection or increased stability of hGH-V2 mRNA. In either case, the finding that levels of hGH-V2 mRNA are controlled independently from hGH-V mRNA suggests that an hGH-V2 protein might have a functional role in gestation.

We have identified low levels of intron 4 retention in both hCS-A and hCS-B transcripts by RT/PCR (Fig. 5). These data suggest that the minor 1.2-kilobase hCS-A mRNA found by others (46) using hCS-A oligonucleotide probe hybridization of placental Northern blots is in fact hCS-A2. A 1.2-kilobase transcript was not detected when an hCS-B oligonucleotide probe was similarly used (46), even though hCS-B2 mRNA is clearly evident by RT/PCR analysis of our stably transfected cell line (Fig. 5). We presume that the hCS-A2/hCS-B2 ratio of 12.6:1.0 in term placentas results in levels of hCS-B2 mRNA which are not easily detectable by oligonucleotide hybridization. Intron 4 sequence analysis of the cloned hCS-A gene (4) and RT/PCR-amplified hCS-A2 cDNA from two placentas differ from earlier reports (4, 28) at three and two positions, respectively (Fig. 6). Our sequence, in agreement with Selby et al. (28), predicts that the open reading frame of hCS-A2 would continue for an additional 104 codons. The resulting hCS-A2 protein would have 80% overall amino acid identity to that predicted for hGH-V2 with 85% identity in the intron 4 region (7). In contrast, the hCS-B2 mRNA, present in much lower concentrations, terminates its reading frame 15 codons into intron 4. If hCS-A2 is translated and functionally important, then hCS-B might represent a mutated form of hCS whose expression levels are gradually diminishing through evolution.

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Fig. 5. Identification of alternatively spliced hCS-A and hCS-B transcripts retaining intron 4. A, schematic representation of RT/PCR assays that specifically amplify intron 4 retaining mRNAs from hCS (hCS-2) or hGH-V (hGH-V2) transcripts. TP-end-labeled (*) sense oligonucleotide primers were located in the region of mRNA encoded by exon 2. The antisense oligonucleotide primers were located in intron 4 (triangular insets). Predicted band sizes and the location of specific endonuclease restriction sites are indicated. The assay does not discriminate between hCS-A and hCS-B gene products. B, oligonucleotide primers specific for hCS-2 amplify a fragment of the predicted 420-bp size from both placental villous RNA and a stably transfected hCS-B expressing cell line (CM-hCS-B). There is minimal evidence of cross-amplification of hGH-V2 transcripts in a stably transfected hGH-V expressing cell line and no nonspecific background bands in RNA isolated from human reticulocytes. The positions of size markers (shown at the left side of the gel) were derived from analysis of pGEM3 digested with HindIII. C, analytical restriction digests confirm the identity of hCS-2 cDNA fragments from term placenta and transfected cell lines. EcoNI (E) digests only the hCS-2 sequences, and BamHI (B) digests only hGH-V2 sequences. All bands are of the expected length (shown at the right side of the gel), as determined from pGEM-3 digested with HindIII (not shown). D, analytical digestions with BstEII differentiate hCS-A2 and hCS-B2. hCS-A contains a BstEII site within intron 4 which predicts a shortening of the amplified fragment from 420 to 372 bp. In contrast, no BstEII sites exist in the hCS-B gene. The BstEII (Bsv) digestion, as predicted, has no effect on the amplified fragment in the CM-hCS-B cell line. The digestion of term placental RT/PCR fragments demonstrates that greater than 90% of the hCS-2 species are derived from the hCS-A gene.

Fig. 6. Comparison of hCS-A2 and hGH-V2 cDNAs and predicted proteins. The sequences of hCS-A2 and hGH-V2 cDNAs are compared starting in exon 4, 3 codons upstream of the intron 4 junction. Nucleotides that are intronic in hCS-A are in lower case. Nucleotides are numbered (+1), and bases that differ from Selby et al. (28) (*) or Chen et al. (4) (+) are indicated. Both hCS-A2 and hGH-V2 terminate (*** ) in the middle of exon 5, predicting proteins of 230 amino acids compared with 191 when intron 4 is spliced out. Location and directionality of the oligonucleotide primers used for hCS-A2 sequencing are overlined.

Fig. 7. Characterization of hCS-L RNA splicing. Diagram of the unique splicing patterns of hCS-L transcripts. The predominant splicing patterns of the other four genes in the GH cluster are shown on the top line; exons are indicated by the boxes and introns by flanking sequence by the solid line. All hCS-L transcripts contain a 19-base extension of exon 2 into intron 2 caused by a G to A transition that destroys the normal intron 2 splice-donor and activates a cryptic site (+2) 20 bases downstream. This unique hCS-L donor site splices to either of two alternate acceptor sites. The majority (88%) of hCS-L transcripts utilize the downstream acceptor site (ag). A minority of transcripts (12%) utilize an alternative site (ag) only 4 bases downstream of the normal splice acceptor (ag), indicated with an arrow. Both hCS-L splicing patterns maintain the same open reading frame.

Transcripts in the placental villi (Fig. 7). Both use a splice-donor site located 20 bases into intron 2. This site is present but cryptic in the other members of the GH cluster. It is specifically activated in hCS-L transcripts because of a G to A substitution at the first base of intron 2 which destroys the normal splice-donor site. The resulting 3' extension of exon 2, therefore, is unique to hCS-L mRNAs. The predominant
splicing pattern, utilized 88% of the time, joins this donor site to an acceptor site 73 bp within exon 3. This hCS-L mRNA maintains an open reading frame and has been described previously (4). The minor splicing pattern, designated hCS-L', is used 12% of the time and was previously undetected. In this second pathway, the same donor site is spliced to an alternative splice-acceptor site that might be reflected in a diversity of protein patterns that might be reflected in a diversity of protein isoforms. Therefore, despite the close juxtaposition of these genes and their very high level of sequence identity, multiple differences in their patterns of expression generate potential for complex and diverse functional roles.

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