Multiple Origins of Transcription for the Human Placental Lactogen Genes*

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Transcriptional activity of the three human placental lactogen (hPL) genes was compared in vitro and their relation to the hPL RNA species obtained from term placenta was analyzed.

We used in vitro transcription system containing the HeLa cell crude extract as the source for RNA polymerase II and initiation factors. Gene fragments of identical length of 0.96 kilobase pair made from hPL1, hPL2, and hPL4 each contained the 5' flanking sequence of 497 base pairs, the first exon, the first intron, and a portion of the second exon. More than 90% transcripts of the hPL1 template were 470 nucleotides long, indicating that transcription was initiated at the proposed cap site. hPL2 and hPL4 genes generated heterogeneous RNA products of about 430, 470, 520, and 680 nucleotides suggesting that multiple start points were recognized for RNA synthesis in vitro. α-Amanitin sensitivity of transcription indicated that the DNA-dependent RNA synthesis was carried out by RNA polymerase II. These results show that hPL1, hPL2, and hPL4 genes have functional promoters and multiple initiation sites for transcription.

 Primer extension analysis of the 5' termini of hPL RNA isolated from term placenta shows that 82-83% of the transcripts are initiated at a region 29 base pairs downstream from a "TATA" sequence. This origin is observed in vitro for the transcript 470 nucleotides long. An additional upstream initiation region (~53) accounts for 8% of transcripts in term placenta and corresponds to the origin for the in vitro transcript of 520 nucleotides. At least three other sites 15, 23, and 39 base pairs downstream from the major cap site are functional in vitro. The initiation site at 4-40 is utilized preferentially for transcription from hPL3 and hPL4 genes in vitro. We have mapped the different transcription origins on hPL genes.

The genes for GH, PL, and prolactin constitute a multigene family that specifies proteins with related activities (1). There are at least two hGH (2-4) and three hPL (3-6) genes, all clustered at band q22-24 of chromosome 17 (7). hPL and hGH genes show 95% sequence homology in the mRNA coding regions (2), and their protein products share 85% homology in amino acid sequence (8). However, these genes are expressed in a tissue-specific manner, hGH in anterior pituitary gland and hPL in placental syncytiotrophoblasts. As an essential step toward understanding the tissue-specific expression of hPL genes in placenta, we studied the transcriptional pattern of the individual hPL genes.

Transcription of eukaryotic genes is primarily regulated at the initiation step of RNA synthesis. The DNA sequences close to the start point of transcription are proposed to contain at least two signals that function to facilitate transcription initiation. The "TATA" homology or promoter element found between 20 and 30 nucleotides upstream of the cap site on most eukaryotic protein-coding genes is implicated in controlling the accuracy of transcription through site-specific initiation (9-12). Sequences located between 40-100 nucleotides upstream from the cap site have been shown to be important in regulating the efficiency of transcription (13-16). Other conserved sequences are shown to regulate post-transcriptional modifications of the primary transcripts such as splicing (17, 18) and polyadenylation (19, 20).

Earlier studies from our laboratory have shown that hPL and hGH genes specify distinct mRNAs that code for identical mature placental lactogen in term placentas (21, 22). However, there is no convincing evidence for the expression of the hPL gene in placenta. In the absence of distinct sequence variation between hPL1 and the other two hPL genes, it was not possible to detect transcripts specific for hPL1 gene. As promoters play an important role in the regulation of gene expression, we analyzed the functionality of hPL1 promoter by comparing the in vitro transcription pattern of DNA templates from hPL1, hPL2, and hPL4 genes. As the cap site for hPL genes has been assigned only tentatively by sequence analysis (4) we asked whether the transcription origin is unique and the same for all hPL genes. We report that hPL genes harbor multiple initiation sites for transcription which correspond to the 5' heterogeneity of hPL RNA isolated from term placenta.

EXPERIMENTAL PROCEDURES

Preparation of DNA Templates—DNA fragments were prepared from the recombinant plasmids carrying hPL1, hPL2, or hPL3 genes (3, 8) by digestion with appropriate restriction enzymes (Fig. 1) and separation by agarose gel electrophoresis. The bands of interest were localized by UV fluorescence after staining with ethidium bromide, transferred onto DEAE-cellulose strips, and eluted as described (23).

In Vitro Synthesis and Purification of RNA—RNA was transcribed from the template DNA according to the procedure of Manley et al. (24) and Weil et al. (25) as modified by Tsai et al. (12). A standard 100-μl reaction mixture contained 12 mM HEPES (pH 7.9), 3 mM MgCl2, 60 mM KCl, 1.5 mM dithiothreitol, 10% glycerol, 0.2 mM...
EDTA, 500 µM ATP, CTP, and UTP, 50 µM GTP, 50 µCi of \([\alpha-32P]\)GTP, 2.5 µg of DNA, and 60 µg of crude extract from HeLa cells.

The HeLa crude extract was prepared by the method of Tsai et al. (12). The reaction mixtures were incubated at 30 °C for 45 min, and RNA was isolated as described (27).

Analysis of RNA by Polyacrylamide Gel Electrophoresis—RNA samples were denatured by suspending in 99% formamide and heating at 100 °C for 5 min and then loaded on a 4% polyacrylamide gel. Electrophoresis was carried out at 20 mA for 4 h (27). Gels were exposed to Kodak XRP-1 film with an intensifier screen at -20 °C for autoradiographic analysis.

Primer Extension Assay—The primer was generated by a two-step restriction digestion of the cDNA clone phPL815. The cDNA insert was released from the pBR322 vector by digestion with PstI and the 768-bp fragment digested with HaeIII and fractionated by polyacrylamide gel electrophoresis to obtain a 143-bp fragment located downstream of the 5' terminus of cDNA. The primer was labeled at the 5' termini with \(\gamma\)-[32P]ATP (Amersham Corp.) and T4 polynucleotide kinase (28) and purified by DEAE-cellulose column chromatography. DNA-RNA hybridization was performed in a final volume of 50 µl containing 0.4 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, approximately 20 ng of end-labeled probe, and 50 µg of placental poly(A+)* RNA (15). The reaction mixture was denatured at 100 °C for 5 min at 68 °C for 16 h. The hybrids were precipitated with ethanol, and extension was carried out in 100 µl of 60 mM NaCl, 10 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 8 mM MgCl₂, 1 mM each of dATP, dGTP, dCTP, and dTTP, 50 µg/ml of actinomycin D, 50 units/ml of RNasin, and 50 units of reverse transcriptase at 37 °C for 1 h (29). The extension products were purified by phenol extraction, ethanol precipitation, and treated with 0.2 N NaOH for 20 min at 68 °C. After dialysis with equal volumes of 10 mM urea, 0.015% xylene cyanol, and 0.05% bromphenol blue and incubation for an additional 15 min at 68 °C, the samples were run on a 6.5% polyacrylamide gel containing 7 M urea at 1700 V for 3 h. End-labeled HaeIII and AluI digestion fragments of pBR322 were run as markers. In order to map the initiation site accurately to the nucleotide, M13 mp8/pUC8 sequence ladder was prepared according to the method of Sanger and Coulson (30).

RESULTS

Transcription of hPL DNA in Vitro—Plasmid DNAs containing hPL₁, hPL₀, or hPL₄ genes were digested with suitable combinations of restriction enzymes to obtain DNA templates for the truncated template assay (Fig. 1). The 1.14-kb gene fragment of hPL₁ contained the 5' flanking sequence of 497 bp, the first exon, the first intron, the second exon, and a portion of the second intron. The 1.4-kb template from the hPL₄ gene contained a 497-bp flanking sequence, the first exon, first intron, second exon, second intron, third exon, and a part of the third intron. The crude extract of the HeLa cells provided RNA polymerase and initiation factors needed for cell-free RNA synthesis.

Electrophoretic analysis of RNA products synthesized in vitro revealed a major radioactively labeled band of about 654 nucleotides for hPL₁, and about 900 nucleotides for hPL₄, corresponding closely to the size expected if transcription starts near the 5' end of exon 1 (Fig. 2). Thus, the hPL₀ gene was shown to contain a functional promoter to specifically initiate RNA transcription in vitro.

In order to confirm that the RNA products synthesized from hPL genes were indeed initiated at the cap site, another set of restriction fragments of 0.96 kb was made from hPL₀, hPL₃, and hPL₄ genes. These DNA templates contained 497-bp flanking sequences on the 5' end of the gene, the first exon, first intron, and a portion of the second exon (Fig. 1). The size of more than 90% of RNA transcribed from the hPL₁ gene matched the predicted length of 464 nucleotides if initiation occurred at the cap site tentatively assigned by sequence analysis of the gene (Fig. 3A). If initiation of RNA synthesis occurred at a site further downstream of the cap site, the expected size could be about 565 nucleotides.

![Fig. 1. Map of the 5' end of placental lactogen genes and their flanking sequences showing the restriction endonuclease cleavage sites.](image1)

![Fig. 2. Autoradiogram showing gel fractionation of 32P-labeled RNA synthesized in vitro from hPL₁ (1.14 kb) and hPL₄ (1.37 kb) templates.](image2)

![Fig. 3. Electrophoretic analysis of RNAs synthesized on 0.96-kb hPL templates.](image3)
Multiple Transcription Origins of hPL Genes

The difference in length of the major RNA transcript from hPL1, hPL3, and hPL4 is not due to differences in the distance from the start site to the 3' termini of templates as all three hPL genes contained the functional promoter for selective initiation of transcription. However, hPL3 and hPL4 genes were shown to harbor an additional initiation site about 39 nucleotides downstream of the tentative cap site, which was preferentially recognized in vitro for the synthesis of RNA (430 nucleotides). Another minor initiation region responsible for the synthesis of the 520-nucleotides-long in vitro transcript occurs 50 nucleotides upstream from the proposed cap site.

In order to elucidate whether multiple initiation sites for transcription observed in vitro are operative in vivo, we determined the 5' end of hPL mRNA isolated from term placenta by primer extension assay. The sizes of extension products were precisely determined with reference to an M13 sequence ladder and end-labeled restriction fragments of pBR322 run in parallel. We used the M13 ladder for the single nucleotide marker system as the cDNA clones for hPL do not extend to the 5' end of the mRNA. As shown in Fig. 4, the 5' termini of the majority of in vitro hPL transcripts (210 nucleotides) are located at 67 ± 1 nucleotides upstream from the PstI site, as has been previously predicted from the sequence analysis. In order to coordinate the numbering of initiation sites observed in vivo and in vitro, this major in vitro initiation site located 29 nucleotides downstream of the TATA box is numbered one (+1). However, the presence of at least four other discrete products of extension reveals that the 5' terminus of the hPL mRNA is not unique. In vivo transcription may also

### Table I

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**Fig. 4. Primer extension analysis of hPL RNA in term placenta.** The 143-bp PstI-HaeIII probe was 5' end labeled and hybridized overnight at 68°C to total poly(A') RNA from term placenta. Extension with reverse transcriptase was carried out as described under "Experimental Procedures." The products were analyzed on 6.5% polyacrylamide, 7 M urea gel. Numbers on the left indicate the size markers. Numbers on the right indicate the length in nucleotides of extension products.

Transcripts from the hPL3 and hPL4 templates resulted in the synthesis of at least four discrete size classes of RNA (Fig. 3B). The major transcript from hPL3 and hPL4 templates was 430 nucleotides long and contributed 42 and 47%, respectively, of the total in vitro transcripts (Table I). Transcripts of 470, 520, and 680 nucleotides from hPL3 DNA accounted for 20, 22, and 15% of transcription. A minor transcript of 800 nucleotides contributed about 1-2% transcription from hPLs DNA.

However, transcription of hPL3 and hPL4 templates resulted in the synthesis of low levels of RNA products of 525 and 680 nucleotides.

The synthesis of the 430-nucleotides-long transcripts from the hPL template each contributed to 21, 20, and 10% of RNA synthesis. A minor transcript of 800 nucleotides contributed about 1-2% transcription from hPL3 and hPL4 DNA. In vitro transcription from hPL DNA also resulted in the synthesis of low levels of RNA products of 525 and 680 nucleotides.

The occurrence of multiple initiation sites for transcription of these genes.

In addition, the absence of a radiolabeled RNA band when DNA was omitted from the assay mixture showed that RNA synthesis under the conditions employed for the in vitro assay is DNA dependent (Fig. 3B, lane 4). Thus our results indicate that the 497-bp sequence located upstream of the proposed cap site of the three hPL genes contained the functional promoter for selective initiation of transcription. However, hPL3 and hPL4 genes were shown to harbor an additional initiation site about 39 nucleotides downstream of the tentative cap site, which was preferentially recognized in vitro for the synthesis of RNA (430 nucleotides). Another minor initiation region responsible for the synthesis of the 520-nucleotides-long in vitro transcript occurs 50 nucleotides upstream from the proposed cap site.

In order to elucidate whether multiple initiation sites for...
be initiated from a region 53 base pairs upstream from the major initiation site generating a 264-bp product. After prolonged autoradiography (3 days at -80°C) the other three minor initiation sites coincide with the location of sites observed with in vitro transcription. The relative amounts of the heterogeneous hPL RNA species were quantitated by densitometric scanning and comparison of the relative intensities of the bands. We estimate that 82–83% of the hPL RNA originates from initiation at the major region at +1. Longer transcripts originating from an upstream start site contribute 8% of hPL RNA, while at least three other size classes are initiated with equal efficiencies at alternative sites on hPL genes (Table 1).

When primer extension was carried out with the same probe and poly(A) RNA from term placenta in the presence of α-32P]CTP, another distinct band, 540 nucleotides in length, was observed (data not shown). This origin is located 329 nucleotides upstream of +1 and corresponds to the start site for in vitro transcript of 800 nucleotides. Less than 0.5% of RNA initiates from this region, and thus this site seems to be responsible for the synthesis of a very infrequent species of hPL RNA. It is possible that some hPL transcripts arise from yet other initiation regions, but their abundance may be too low for detection in comparison to other species of hPL RNA.

The multiple origins of transcription of hPL genes are mapped in Fig. 5. As mapping by primer extension is subjected to an error of 1 or 2 nucleotides, the positions indicated in the map may vary ±2 bases from their actual location in vivo. The location of major transcription origin 29 nucleotides downstream of “TATA” box and 61 nucleotides downstream of “CAAT” box and 61 nucleotides upstream of the translation initiation codon agrees with the spatial arrangement of these conserved regulatory sequences in various well-defined protein-coding genes transcribed by RNA polymerase II and more likely the reason for preferential initiation of RNA synthesis from this site in term placenta.

**DISCUSSION**

Our studies on the cell-free transcription of hPL1, hPL3, and hPL4 genes show that the 500-bp 5’ flanking sequence of these three genes harbors functional TATA sequence to promote faithful transcription. The heterogeneous RNA transcripts generated from hPL and hPL4 templates reveal the occurrence of more than one initiation site for transcription which are utilized with different efficiencies in vitro and in vivo. It has been shown that the efficiency of initiation is influenced by the actual DNA sequences around the mRNA start site (31). The start point for hPL1, 5'-TCCAGCATC-3', matches the consensus 5' PyPyCAPPy-Py-PyPy-3' (32) in 7 out of 9 positions and thus explains its ability to support faithful initiation of more than 90% of transcription in vitro.

For hPL3 and hPL4, the initiation region TCCTGTTGGA resulting in a 430-nucleotide transcript is utilized more frequently than the TCTAGGATC which results in a 470-nucleotide transcript.

The major initiation site mapped by primer extension analysis of placental poly(A) RNA is identical to the first nucleotide of exon 1 as analyzed by sequencing of the hPL genes (start site +1). However, in vitro transcription originates primarily from a different site at +40.

In vitro transcription of genes by RNA polymerase II is generally found to be influenced by sequences localized within 20 to 34 nucleotides upstream of the cap site but insensitive to sequence elements 5' to the TATA box (33). Maximal initiation of in vitro transcription from site +1 on hPL genes could be explained in terms of the influence of sequence elements upstream of the TATA box. The spatial arrangement of regulatory sequences with respect to the mRNA cap site is highly conserved and critical for transcription in vivo (34). Transcription of globin genes in vivo requires the CAAT box at about 70 bp 5' to the mRNA cap site, and a deletion between these two regions results in a 10-fold decrease in transcription of β-globin gene (35). Alteration of the distance between the TATA box and the initiation site in the rabbit β-globin gene shifts the initiation site to a point about 30 nucleotides from the TATA box (33). Also the number of bases between the capping site and the initiator ATG is relatively constant (50–53 bp) among the β-like globin genes (34). As seen from Fig. 5, the initiation site +1 is located at an optimal distance in hPL genes from the CAAT box, TATA box, and the translation initiation ATG codon and thus accounts for its preferential utilization for transcription in vivo. An AT-rich sequence (ATAAAT) resembling the “TATA” box is also present 30 bp upstream of the minor initiation site at +53 which contributes to ~1% of the transcripts in vivo suggesting that the presence of TATA or a TATA-like element at a suitable distance from the initiation site facilitates transcription.

The heterogeneous hPL RNA species observed in term placenta by primer extension assay reflect the mixture of transcripts from all expressed hPL genes. As heterogeneous RNA products are formed from hPL1 and hPL0, DNA in vitro, it is more likely that hPL RNA classes observed in vivo originate from multiple initiation sites on these genes. It is also possible that transcription starts from a single site on different members of hPL multiple genes. However, in the absence of specific probes to differentiate the individual hPL gene products, we are not able to correlate the various in vivo transcripts to specific hPL genes.

Analysis of the in vitro transcriptional pattern of the hPL4 gene revealed that it has a functional promoter to support specific initiation. Also the predominant initiation site recognized in vivo on hPL4 gene corresponds to the major in vivo transcription site. However, sequence analysis of hPL1 has

**Fig. 5. Nucleotide sequence of the 5' flanking region and a portion of the first exon of hPL genes showing multiple origins of transcription.** The “CAAT” consensus, “TATA” sequence, “TATA-like” sequence, and the translation initiation codon are underlined. The origins of transcription that are functional in vivo are indicated by solid arrows pointing downward. Arrows pointing upward show origins mapped in vitro. The sequences are numbered from the major in vivo initiation site +1.
shown the occurrence of a point mutation in the consensus of the 5′ splice site of the second intron which converts GT → AT. This kind of mutation in the human β-globin gene has been shown to abolish normal splicing at this site and lead to the formation of aberrant splice products (36, 37). hPL has a deletion of 2 bases in the consensus “CAAT” box compared to hPLl and hPL4. Deletion in this region of the globin gene drastically reduces the efficiency of transcription (13). hPLl also shows many single base differences in the promoter region (48). Thus transcription initiation from positions other than the canonical cap site is also utilized in a stage-specific manner. Various cap-site elements might be utilized in a tissue-specific manner. Thus transcription initiation from positions other than the canonical cap site may be a common phenomenon, and probably different sites might be utilized in a stage-specific or tissue-specific manner. Though their functional significance is not fully understood, multiple initiation sites may respond to different control signals or play an important role in governing the ultimate abundance of the gene product within the cell.

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