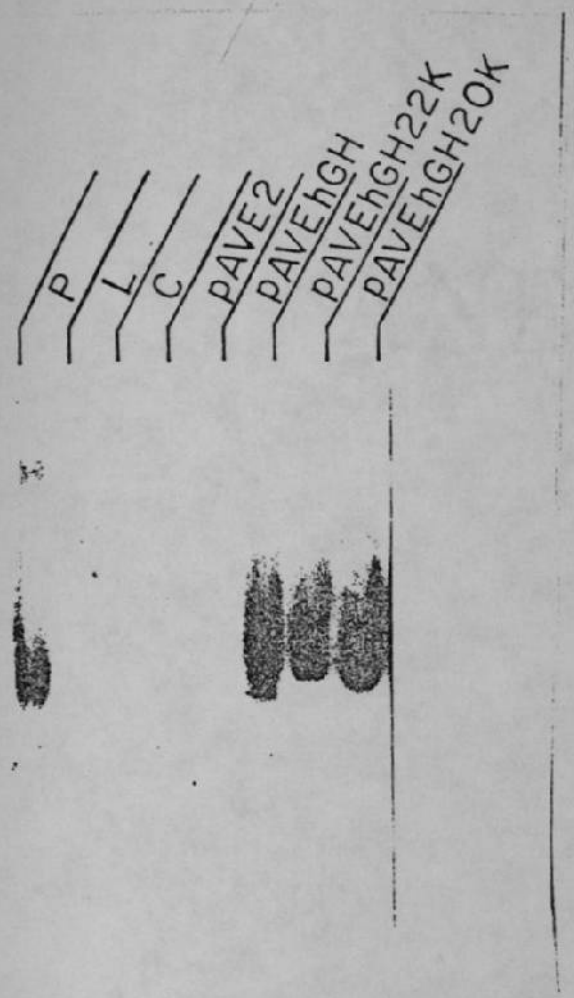

Figure 3

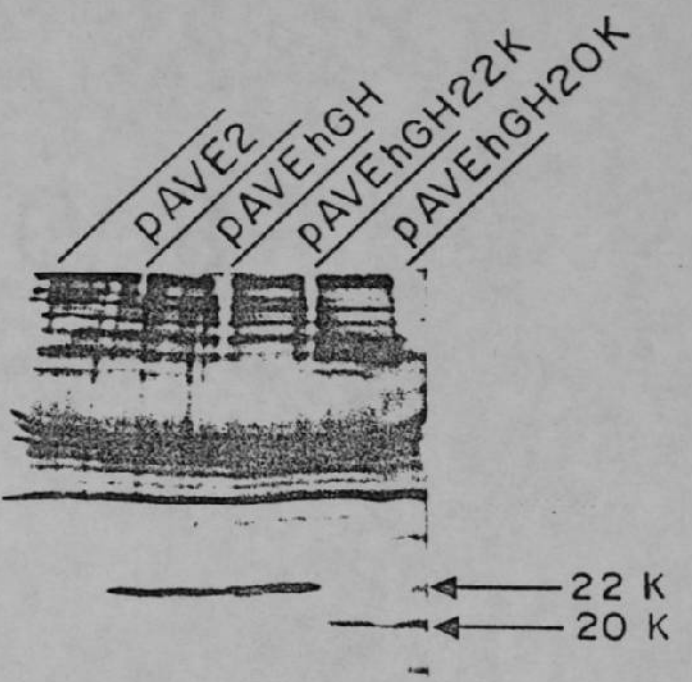

Figure 4

ANEXO B

Coding potential of transfected human placental lactogen genes

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ABSTRACT

We have joined the promoter-less sequences of the three hPL genes (hPL-1, hPL-3 and hPL-4) to strong transcriptional control elements. *in vivo* ^{35}S-labeled proteins from the culture medium of cells transfected with the genes were resolved on SDS-polyacrylamide gels. The presence of characteristic labeled bands, visualized by autoradiography, determined that hPL-4 and hPL-3, but not hPL-1, contribute to the production of mature hPL. In these experiments hPL-3 expressed more RNA and protein than hPL-4. By exchanging the first two exons among hPL and hGH genes, we determined that the abundance of chimeric proteins depended on the genetic origin of the first two exons. Finally, we found evidence indicating that the splice mutation (G→A) at the beginning of the second intron of hPL-1, is not the only cause of the apparent lack of inactivity of this gene, since its reversion does not restore expression.

INTRODUCTION

The human growth hormone-placental lactogen gene family is a multigene complex containing two human growth hormone (hGH) and three human placental lactogen (hPL, also known as chorionic somatommamotropin; hCS) genes (1,2). The entire gene cluster is located on the long arm of human chromosome 17 at bands q22→24 (3). The genes display the following 5' to 3' arrangement: hGH-N, hPL-1 (or hPL-L, L for like), hPL-4 (also known as hCS-A or hCS-1), hGH-V and hPL-3 (also named hCS-B or hCS-2). The best characterized protein products of this family are the secreted hGH and hPL polypeptides containing 191 amino acids. They are produced in the pituitary gland and placenta, respectively.

Recombinant DNA analysis has revealed a paradox in the coding potential of the hGH and hPL genes. The hGH-N gene, both *in vivo* and *in vitro*, generates through differential splicing of its primary transcription product, 22 kDa (90%) and 20 kDa (10%) forms of hGH (4,5). The hGH-V gene, whose expression has been demonstrated only in the placenta and in a single human pituitary tumor (6), has recently been confirmed to also generate *in vitro* a 22 kDa form. However, no 20 kDa protein derived from this gene has been detected (7). cDNA cloning and DNA sequencing also have revealed the existence in placenta of a second type of hGH-V mRNA. By retaining an in-frame fourth intron, this new mRNA is predicted to encode a mature protein of 26 kDa (8).

A completely different situation is observed with the hPL genes. The hPL-4 and hPL-3 genes have been found to be active in term placenta. In addition, their cDNAs have been cloned and sequenced (9). Their mRNAs are slightly divergent in nucleotide sequence. The encoded pre-hormones of these two mRNAs differ in a single amino acid position within the signal peptide (at position −24, hPL-3 codes for alanine while hPL-4 codes for proline). Yet, the mature hormones are identical. The third gene (hPL-1), is presumably nonfunctional, since it contains a mutation (G→A) at the 5' or donor splice site of the second intron. Transcripts derived from it have not been detected (9, 10). Therefore, while two hGH genes generates at least four different hormones, the sequences of the three hPL genes predict the synthesis of a single form of mature hPL hormone.

The sequence of both hPL-3 and hPL-4 genes and their cDNAs, predict that they might contribute to the placental production of hPL. However, since the mature proteins expected to be derived from them are identical, it is impossible to distinguish their gene(s) of origin. No evidence has been obtained demonstrating that the expression of either gene actually specifies the mature hPL protein.

In this study we performed an analysis of the *in vitro* expression products of all hPL genes. We specifically addressed the question of whether or not the hPL-3 and hPL-4 genes, known to be transcriptionally active in term placenta, produce mature hormones. These two genes, at different expression levels, were found capable of producing an intrinsic hPL protein. Experiments were also designed to determine if the splice point mutation at the beginning of the first intron of the hPL-1 gene, is the only cause of its apparent lack of expression.

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MATERIALS AND METHODS

Recombinant DNA constructions and preparation of plasmid DNA

Restriction and other enzymes were obtained from commercial suppliers and used according to their manufacturers instructions. The isolation of hGH and hPL genes have been previously reported (11). pNUT, constructed by R. Palmiter *et al.* (12) which already carries the hGH-N structural gene in front of the metallothionein promoter, was a generous gift. The hPL genes cloned in pSV2gpt (13) were kindly provided by G. Saunders. DNA restriction fragments were purified from preparative agarose or polyacrylamide gels. This was performed by electroelution, or by glass bead extraction (14) from the agarose gel slices. hPL and hGH promoter-less genes were subcloned into pNUT. The large *Bam*HI to *Eco*RI fragment of pNUT, was ligated to DNA fragments carrying the genes of interest. The genes consisted of sequences from their naturally occurring *Bam*HI site (except hPL-1; see Results) at nucleotide +2, to a natural or artificial (linkers) *Eco*RI site located several hundred nucleotides downstream of the polyadenylation signal. To construct our negative control, pNUT(−), we took advantage of the presence, in pNUT, of two *Xma*I sites. They flank hGH coding sequences: one artificial site is present at position −4, while the other is a natural site located four nucleotides downstream of the termination codon. By cutting with *Xma* I, diluting and ligating back, we obtained the derivative of pNUT lacking the hGH structural gene: pNUT(−).

Ligations, bacterial transformations and plasmid DNA isolation and characterization were carried out using standard protocols (15). Recombinant plasmids carrying all hGH, hPL or hybrid genes were characterized by digesting their DNAs with several diagnostic enzymes, by Southern blotting (16) or nucleotide sequencing (17).

Cell culture, DNA transfection, isolation of RNA and labeling of secreted proteins

COS-7 cells (a gift from T. Kuo) were adapted to grow in Dulbecco's modified Eagle's medium (Sigma chemical Co, St Louis MO.) containing 1% fetal calf serum (FCS), (Hyclone Laboratories, Inc. Logan, Utah). They were maintained at 37°C with 5% CO_2. By lowering the FCS concentration we could precipitate and analyze larger volumes of media. Plasmid DNA (7.5 μg/ 25 cm^2 culture flask) was transfected by the calcium phosphate method (18). We evaluated transfection efficiency performing CAT assays or through radioactivity counting of RNA hybridizated with the DHFR probe in slot blots. The CAT assays were carried out on a fraction of cultured cells or the entire culture co-transfected with both the test plasmid and pCMVCat (19).

Total RNA was recovered by the guanidinium thiocyanate-phenol-chloroform technique (20). Quantity and quality of RNA preparations were determined spectrophotometrically and corroborated by agarose gel electrophoresis (15).

To label newly synthesized and secreted proteins, 48 h after cells transfection the previously mentioned medium was replaced for a methionine-free medium containing 1% dialyzed FCS and ^{35}S-methionine (Amersham Intl, Buckinghamshire, England). *In vivo* labeling of newly synthesized proteins was performed by extending the incubation period for an additional 4 h. We labeled with 12.5 μCi of ^{35}S-methionine per ml of medium. The incubated medium was removed from culture flasks and stored. Since, the genes under study code for secreted proteins, we recovered their expressed products from 150 and 300 μl aliquots of the media by precipitating twice with four volumes of cold acetone. Subsequently, we dissolved the recovered proteins in layering buffer for SDS-polyacrylamide gel electrophoresis (21).

Southern blotting, Northern analysis, visualization of labeled proteins and radioimmunoanalysis

^{32}P-dCTP was purchased from Amersham Intl. (Buckinghamshire, England). Hybridization of DNA in nitrocellulose membrane was carried out as described by Southern (16). RNA was denatured and resolved according to size by agarose gel (22) electrophoresis. Once the above was performed, they were transferred to nitrocellulose sheets and hybridized to the probe (23). Both hybridization techniques used as probe, a 550 bp *Hae*III fragment of hPL cDNA (24) labeled with ^{32}P-dCTP by the technique of random primers (25).

Protein samples dissolved in layering buffer were boiled for 2 min and applied to 5−13% discontinuous polyacrylamide gels (21). Gels were placed on filter paper and dried under vacuum at 80°C. The dried gels were exposed to X-ray films at room temperature. Quantification of hGH was achieved using a commercially available hGH radioimmunoassay kit (Diagnostic products Co., Los Angeles, CA).

RESULTS

A new set of high expression plasmids for hPL structural genes

pNUT, contains the SV40 enhancer and metallothionein promoter directing the transcription of the promoter-less hGH-N gene (figure 1A). In addition, it efficiently expresses hGH in cell culture (12). We found, by radioimmunoassay, that COS-7 cells transfected with pNUT by the calcium phosphate method (18), yielded extracellular hGH values averaging 700 ng per 25 cm^2 culture flask.

We transferred the structural sequences (promoter-less) of all the hPL members of the hGH-hPL multigene family into pNUT (see figure 1B). This was accomplished by simply replacing the hGH-N gene structural sequences present in pNUT, for the corresponding sequences of the hPL genes. However, because hPL-1 gene lacks the convenient *Bam*HI site used for the transfer, we constructed a hybrid gene between hPL-1 and hPL-3 genes to provide it with such a site. The hybrid consists of the *Bam*H1−5′ end flanked first exon, first intron, and part of the second exon of the hPL-3 gene. The rest consists of hPL-1 sequences from the *Pvu*II site, within the second exon, to the *Eco*RI site at the 3′ end of the gene. Having constructed this hybrid allowed us to not only gain the useful *Bam*H1 site, but also allowed us to retain intact the second exon/second intron boundary of hPL-1. This area includes the donor splice mutation of interest, previously identified as potentially being the cause of lack of hPL-1 gene expression. From here on, this hybrid gene will be used instead of the hPL-1 wild-type gene. The recombinant plasmids were characterized by digestion with restriction enzymes (figure 1C) and by Southern blot analysis (figure 1D).

Expression of transfected hPL genes at the protein level

The figure 2 autoradiography reveals that cells transfected with the plasmid carrying the hGH-N structural gene (pNUT), secreted characteristic 22 kDa and 20 kDa forms of hGH (lane: hGH-N). The lane containing media from pNUThPL-1 transfected cells (lane: hPL-1), does not exhibit bands of at least the same intensity

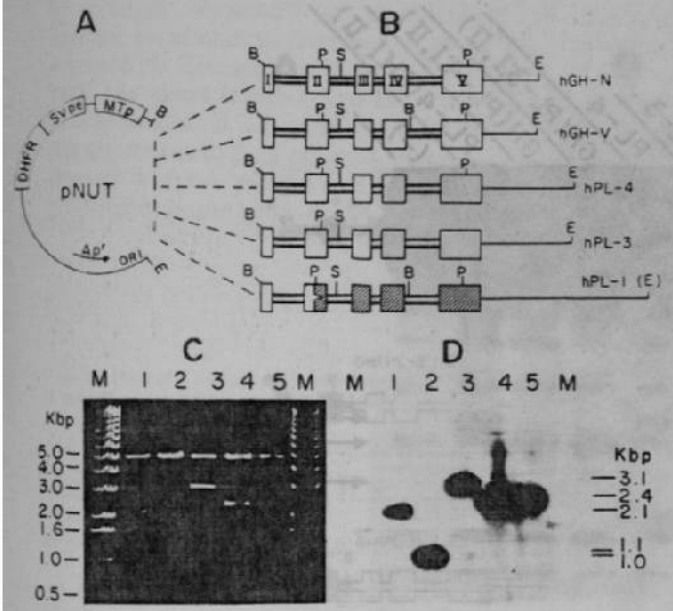

Figure 1. Construction of the hPL and hGH expression plasmids. To subclone hPL genes into pNUT(A), we replaced the hGH-N gene sequences in pNUT for the corresponding ones of hPL genes (B). The drawings at the top illustrate the maps of the expression plasmids (A and B). Both, restriction enzyme (C) and DNA hybridization (D) analysis, confirmed the identity of the new expression plasmids. Lanes in C and D show gel and Southern autoradiography of plasmids cut with *Eco*RI plus *Bam*HI and are as follows: 1, pNUT; 2, pNUThGH-V; 3, pNUThPL-1; 4, pNUThPL-3; and 5, pNUThPL-4. M corresponds to molecular weight (in kbp; at the left of C) DNA standards. Only the sizes of hybridizing bands are indicated (in kbp at the right of D). The hPL-1 is really a hybrid of hPL-3 and hPL-1 (see Materials and Methods section for explanation). The pNUThGH-V construct, presented here, was not analyzed. MTp=mouse metallothionein promoter; SVpe=SV40 early promoter; DHFR=structural gene for dihydrofolate reductase; Ap^r=B-lactamase gene; ORI=pBR322 origin of replication; B=*Bam*HI; P=*Pvu*II, S=*Sac*I, E=*Eco*RI. Boxes represent exons.

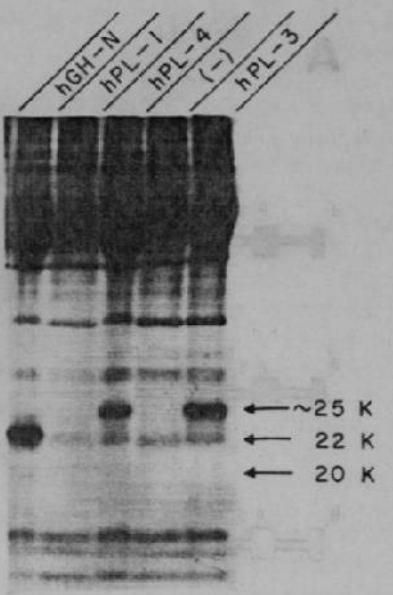

Figure 2. *In vitro* production of secreted proteins by hGH-N and hPL genes. Media from COS-7 cells transfected with each of the plasmids and incubated in the presence of ^{35}S-methionine, was analyzed by discontinuous SDS-polyacrylamide gel (5%−13%) electrophoresis and autoradiography. The gene present in each plasmid used for transfection is indicated at the top. The (−) symbol identifies the media from cells transfected with the vector alone [pNUT(−)]. Sizes of characteristic hPL and hGH bands are indicated in kDa (K) at the left.

and size of hGH. Media from cells transfected with pNUThPL-4 (lane: hPL-4) presents a less prominent band, but of slightly greater size (close to 25 kDa) than that of hGH. Finally, the only hPL-3 form observed (lane: hPL-3), is of hPL-4 size. However, its intensity is that of the 22 kDa form of hGH.

Differences in the expression of hPL proteins

As noticed above (figure 2), while the hGH-N gene gives rise to a prominent band of approximately 22 kDa, hPL-3 and hPL-4 genes express proteins of slightly greater size (~25 kDa). Furthermore, and consistently throughout several independent experiments, the hPL-4 band always appeared weaker than the hPL-3 band. We were interested in investigating the cause of such heterogeneity in the expression levels of these genes.

To approach this problem, we chose to study the cell culture production of extracellular hPL-hGH chimeric proteins resulting from the transient expression of a new hybrid gene pair. These hybrids possess the first two exons from hPL-3 or hPL-4 genes, and sequences of hGH-N gene that conform the remaining part of their structure. We named these hybrids GH(PL-3:I,II) and GH(PL-4:I,II) respectively (see map in figure 3A). By comparing both hybrid genes, differences were observed in the expression levels of GH(PL-3:I,II) versus GH(PL-4:I,II) chimeric proteins [figure 3B; compare lanes labelled GH(PL-3:I,II) and GH(PL-4:I,II)]. Same results, we might add, as with the proteins derived from normal non-hybrid hPL-3 and hPL-4 genes [see lanes labeled hPL-3 and hPL-4 in figure 2 and labeled PL-3 and PL-4 in figure 3B).

Consistently, we obtained less protein when sequences from the first two exons of hPL-4 gene were present. On the contrary, we detected more hGH-like protein when the first two exons were from hPL-3. Moreover, when the last three exons were from hPL-4 gene, and the first two exons were from hGH-N (see map of this chimeric at bottom of figure 3A), the band intensity resembled that of hGH-N protein [figure 3B, lane PL-4(GH:I,II)]. Therefore, relative abundance of hPL protein products is a function of the first two exons.

To further investigate the different levels of *in vitro* expression observed for these active hPL genes, we carried out estimations of the relative abundance of their RNA transcripts. Using slot blot analysis, we found approximately ~8-fold more RNA hybridizable to our probe from the total RNA isolated of cells transfected with the hPL-3 structural gene sequences, as compared to hPL-4 (figure 4). This same result was observed even when only the two first exons of hPL-3 were contributing to a hybrid gene (D.R.-P. and H.A.B.-S., submitted. Therefore, the higher observed hPL-3 protein expression, seems to be a consequence of having more RNA derived from the hPL-3 gene.

Dissecting the putative hPL-1 pseudogene

Next we, decided to test if the donor splice site point mutation at the second intron of the hPL-1 gene, was the only cause of the apparent inactivity of this gene. Comparing nucleotide sequences at the second exon/second intron border area, among the active hPL-3 gene and the putative hPL-1 pseudogene, revealed that we could easily exchange this region between these two genes. We found *Pvu*II and *Sac*I sites located 30 bp upstream and 86 bp downstream respectively, from the mutation site (figure

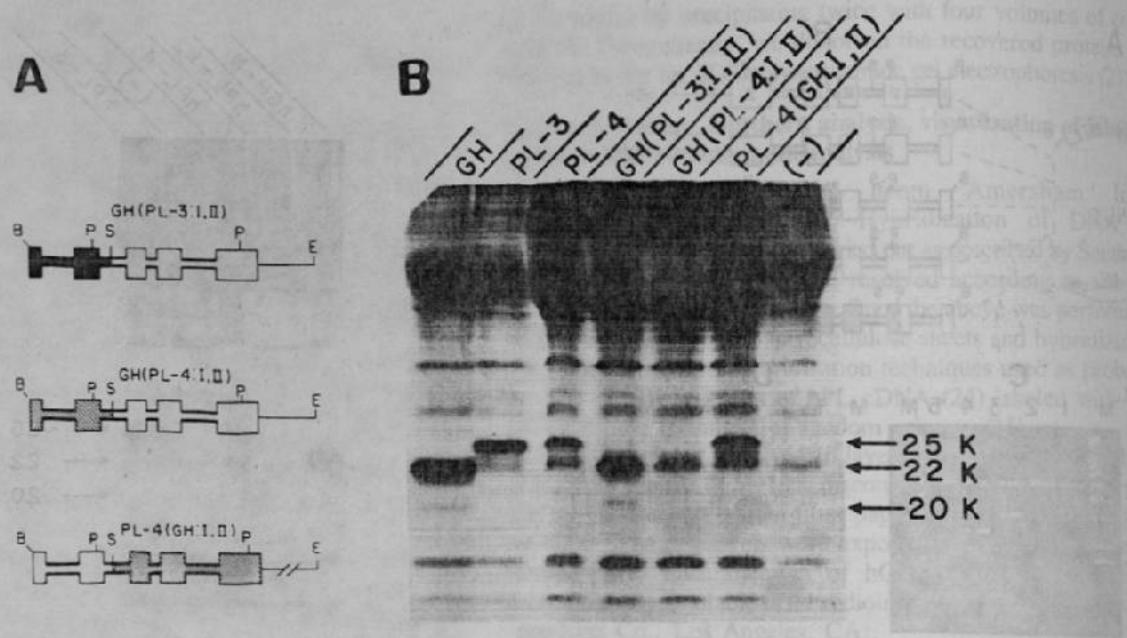

Figure 3. Diversity in size and abundance of protein expression products from, hPL and recombined hPL-hGH genes. Maps in A demonstrates the structure of the hybrid genes; their are composed of hPL-3 (solid) or hPL-4 (hatched) and hGH (open) gene portions. As in the previous figures, dealing with protein analysis, each lane in B corresponds to media of cells transfected with plasmids carrying the genes indicated at the top. The negative control, (−) symbol, distinguishes the media from cells transfected with pNUT(−) (vector alone). The new bands are pointed by arrows. Size is also in kDa (k).

1B). In addition to the splice site point mutation, this region flanked by *Pvu*II and *Sac*I of approximately 120 bp, differs within these two genes in only three nucleotide positions. These differences, are located inside the intron at nucleotide positions of little importance for the pre-mRNA processing (26). The *Pvu*II-*Sac*I region encompassing the second exon/second intron boundary, was exchanged between the putative hPL-1 pseudogene and the active hPL-3 gene. This manipulation gave rise to two new hybrid genes. We named these new recombinants as hPL-1r and hPL-3m, for 'repaired' hPL-1 and 'mutated' hPL-3 genes, respectively.

To determine the effect on the splice mutation expression, we transferred these hybrid genes, once constructed, into pNUT. Both the hybrid and non-hybrid genes (controls) were introduced into COS-7 cultured cells. Figure 5B demonstrates the results obtained from the analysis of *in vivo* labeled secreted proteins. In lane labeled PL-3 which corresponds to media of cells transfected with pNUThPL-3, we easily detected an hPL band. On the other hand, cells transfected with either pNUThPL-1 (lane: PL-1) or pNUThPL-3m (lane: PL-3m), revealed the absence of obvious hPL bands. Likewise, we could not observe an hPL band from the media of cells transfected with the new repaired hPL-1 gene (figure 5B, lane: PL-1r).

A dramatic effect of the splice donor site mutation at the second intron of hPL-1 was also observed; when, we constructed and used in comparative studies, a new hybrid gene. It consists of hPL-3m sequences, joined at the unique *Sac*I site (within the second intron) to the remaining portion of hGH-N gene (bottom of figure 5A). This new hybrid was studied in conjunction (as positive control) with the previously mentioned hybrid gene created between hPL-3 and hGH-N.

A prominent hGH-like (similar in hGH size) protein was found in the media of cells transfected with the non-mutant hybrid gene [see map of GH(PL-3:I,II) in figure 5A, and expression results in lane: GH(PL-3:I,II) of figure 5B]. This positive control gene

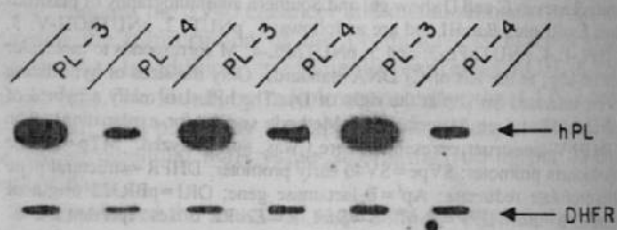

Figure 4. Slot Blot analysis of hPL-3 and hPL-4 RNA expressions. Total cellular RNAs were obtained from three independent experiments in which COS-7 cells were transfected with either pNUThPL-3, or pNUThPL-4. 3μg of RNAs were applied to the slots and hybridized with a DHFR cDNA probe. To determine efficiencies of transfection, the slots were cut and its radioactivity contents measured by liquid scintillation counting. RNAs in amounts compensating transfection efficiencies, were then hybridized separately with the DHFR and with an hPL cDNA probe (arrows). Extent of hybridization was also assessed by counting radioactivity in each slot.

also carries the alternative splice acceptor site present inside exon 3 of the hGH-N gene (4). Splicing exons 1 and 2 to this alternative acceptor site, results in the 15 amino acid internal deletion characteristic of the 20 kDa form of hGH. The presence of a new minor band of 20 kDa together with the 22 kDa band, in the media of cells transfected with pNUTGH(PL-3:I, II), demonstrates that the alternative splicing mechanism also occurs in this hybrid gene.

No hPL or hGH-like proteins were observed (last lane in figure 5B) when the second member of this pair carrying the hPL-1 mutation was used in the transfection.

Effect of the splice mutation of hPL-1 gene in mRNA production

We wanted to know whether the apparent absence of hPL protein from cells transfected with the pNUThPL-1 plasmid, was a

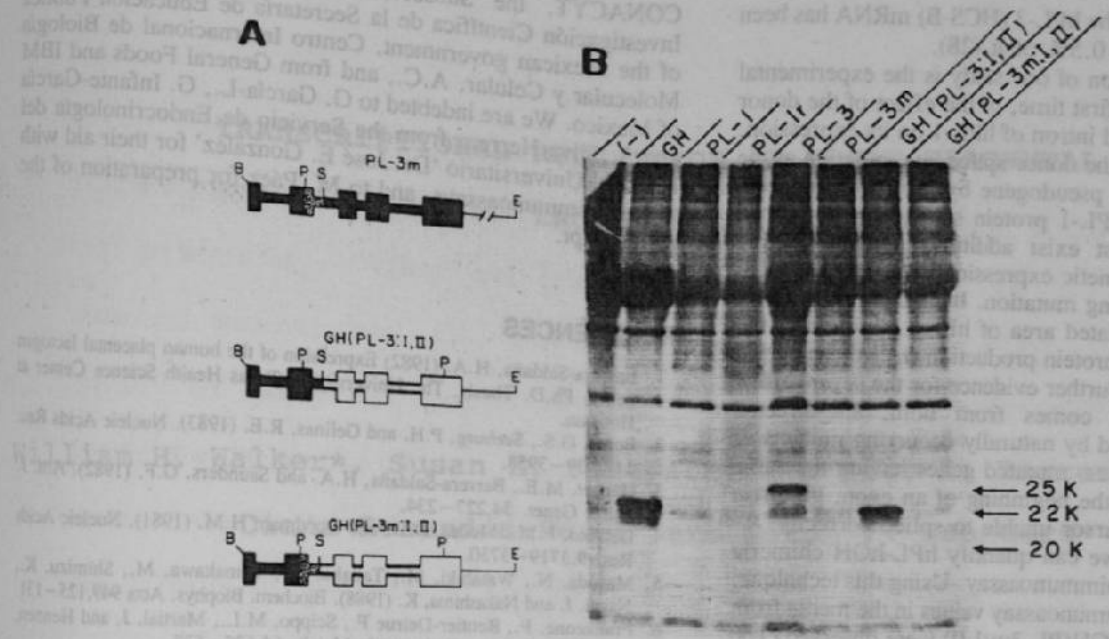

Figure 5. Effect of the donor splice site mutation of hPL-1 gene on the production of secreted proteins. Cells transfected with the different plasmid carrying the hybrid genes indicated at the top, were analyzed for the presence of secreted labeled proteins in their media. Hybrid genes were constructed as described in the Material and Methods and Results sections, and for purpose of clarification, maps of key hybrid genes are shown in A. Characteristic non-chimeric and chimeric proteins were visualized as described in Material and Methods section, gene portions in black, hPL-3 gene; stippled, hPL-1; and open, hGH. Only important recognition sites for restriction enzymes are indicated, B=*Bam*H1, P=*Pvu*II, S=*Sac*1 and E. *Eco*RI. Arrows in B indicate the size in kDa (k) of the new proteins originated from plasmids transfected. (−)=media from the negative control cells.

consequence of having no RNA derived from the PL-1 gene. We performed a Northern blot analysis (23) of the total RNA extracted from transfected cells. We included as a positive control, RNA isolated from human placenta and pituitary gland. In addition, as another positive control, we included RNA from cells tranfected with the plasmid carrying the hPL-3 structural sequences. The results of this analysis are presented in figure 6. Cells transfected with the plasmid carrying the hPL-1 gene lacked hPL specific RNAs (figure 6, lane: PL-1). We observed a dramatic reduction in the hybridizable RNA content from cells transfected with the plasmid carrying the hPL-3 mutated gene (figure 6, lane: PL-3m); as compared with, the wild type hPL-3 gene acting as control (lane: PL-3). Finally, when using pNUThPL-1r, we unexpectantly observed only a faint reappearance of hPL mRNA (figure 6, lane: PL-1r).

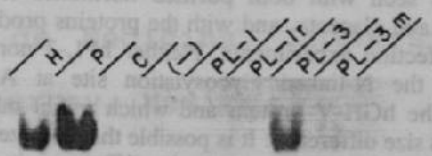

Figure 6. RNA expression effect of the donor splice site mutation of hPL-1 gene. The figure demonstrates the results of the Northern blot analysis (23) practiced to total RNA (10 μg), isolated from cultured cells transfected with the indicated (at top) plasmids. H and P represent lanes containing total RNA from human pituitary gland (2 μg) and placenta (3.8 μg), respectively. The (−) symbol correspond to RNA (10 μg) isolated from cells transfected with the pNUT (vector alone). Lane labeled C represents total RNA of mock-transfected cells.

DISCUSSION

DNA cloning and sequence studies have lead to the isolation of cDNA clones for all hGH and hPL genes except hPL-1. However, experiments directed to demonstrate that each of the mRNAs corresponding to the identified cDNAs indeed end up as proteins, have been few for hGH (7, 27) and none for hPL genes. The reintroduction of cloned genes into cultured cells, by DNA transfection (18), is a valuable method to identify and dissect sequences required for gene function and their mutations. We chose this approach to determine the coding potential of all hPL genes. To achieve our objective, we forced the *in vitro* expression of all the hPL promoter-less genes, by joining them to the strong heterologous transcriptional control sequences present in pNUT (12).

The new results of the present study demonstrate for the first time that the hPL-4 and hPL-3 genes, but not hPL-1, contribute to the production of mature hPL. Here we also demonstrate that in spite of being highly similar, the structural sequences of these genes respond differently to the same heterologous promoter. Each of these two genes give rise to one protein. Although the secreted proteins expressed by these genes have identical amino acid sequence, they differ at their expression level. As a consequence of this finding, we designed exon exchange experiments to gain new insights in the understanding of this phenomenon. The same result was seen when only the two first exons of the genes were contributing to a hybrid gene. The observed higher hPL-3 protein expression seems in part to be a consequence of having more RNA expression from the hPL-3 gene sequences. This *in vitro* findings do not resemble what occurs *in vivo*, while the hPL-4 mRNA (HCS-A) accounts for

3% of the placenta mRNA, the hPL-3 (HCS-B) mRNA has been estimated to represent only 0.5% of it (28).

Finally, a third contribution of our study is the experimental demonstration, also for the first time, of the effect of the donor splice mutation at the second intron of hPL-1 on its expression. In spite of having replaced the donor splice site mutation at the second intron of the hPL-1 pseudogene by normal sequences, we could not observe an hPL-1 protein secreted into the cell medium. Thus, there must exist additional mutations that contribute to this lack of genetic expression. There is no doubt of the severity of this splicing mutation. Indeed we prove here that by introducing the mutated area of hPL-1, into either the hPL-3 or the hGH-N gene, protein production from these hybrid genes is severely reduced. Further evidence for the severe effect of this type of mutation comes from both, site-directed mutagenesis studies (26) and by naturally occurring mutants of β-globin genes. In both cases, mutated genes having the same change of a G for an A at the beginning of an exon, give rise to a messenger RNA precursor unable to splice correctly.

We recently found that we can quantify hPL-hGH chimeric proteins using an hGH radioimmunoassay. Using this technique, non-detectable hGH radioimmunoassay values in the media from cells transfected with pNUTGH(PL-3m:I,II) were observed. The media of cells corresponding to the control experiment, where GH(PL-3:1,II) gene was used, gave hGH RIA values of about half of those of pNUT. Five-fold lower RIA values were found for the expression of GH(PL-4:I,II) as compared to GH(PL-3:I,II) (D.R-P., and H.A.B-S, submitted).

We have no explanation for the difference in size observed in the electrophoretic analysis of hGH and hPL proteins. The difference was seen with both purified hormones from the pituitary gland and placenta, and with the proteins produced in the gene transfection experiments. Neither hPL-3 nor hPL-4 proteins have the N-linked glycosylation site at Asn-140, predicted for the hGH-V protein and which might otherwise account for this size difference. It is possible that the size change observed, may simply be accounted for by differences in protein-SDS interactions.

In conclusion, our analysis of the *in vitro* expression products of hPL genes demonstrates that hPL-3 and hPL-4 have the potential for contributing to mature hPL. It also provides evidence indicating that the hPL-1 gene has accumulated severe mutation(s), other than the donor splice site deffect at its second intron. Furthermore, through our study, we have identified the gene region between the capping site and the second intron as the origin of differences in expression levels seen here for hPL-3 and hPL-4. The results obtained with the hPL-hGH genes hybrids corroborate and strengthen our findings.

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ABBREVIATIONS

bGH, bovine growth hormone; bp, base pairs; CAT, chloramphenicol acetyl transferase; FCS, fetal calf serum; hCS, human chorionic somatomammotropin; hGH, human growth hormone; hPL, human placental lactogen; hPrl, human prolactin; kbp, 1000 base pairs; kDa, 1000 daltons; Pre-hPL, hPL precursor; SV40, simian virus 40; SDS, sodium dodecyl sulphate.

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TRANSCRIPTIONAL REGULATION OF HUMAN PLACENTAL LACTOGEN GENES

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The placenta is an important source of a diverse group of hormones, cytokines and growth factors necessary for continuation of pregnancy. Due to its continued growth and differentiation during gestation, the placenta represents a unique organ for the study of hormonal genes during development. The production of one hormone in particular, placenta lactogen (PL) also called chorionic sommatomammotropin (CS), reflects the development of the placenta as blood PL levels rise throughout pregnancy peaking at term.

Human placental lactogen (hPL) is a member of a closely related gene family that includes growth hormone (hGH) and prolactin (hPRL). Though these genes are highly similar and have evolved from a common precursor they have dramatically different physiological functions and regulatory mechanisms. Recently, advances have been made in understanding the regulation of hPL production and its physiological significance. In this review we discuss the various physiologic actions of hPL during pregnancy, the evolution and structure of hPL genes and, the tissue specific regulation of hPL gene expression.

Structure and Production of hPL Protein

Human placental lactogen (hPL), also called chorionic somatomammotropin, is a single-chain polypeptide hormone of 22,000 Da. It is composed of 191 amino acids with two intramolecular disulfide bridges (1) but contains no carbohydrate residues (Fig. 1). The mature hormone is derived from a precursor of

25,000 Da, that has a 26 amino acid signal sequence cleaved from its amino terminal end (2). Josimovich and MacLaren (3) first defined human placental lactogen as a polypeptide hormone present in extracts of human term placentae and retroplacental blood, that exhibited both potent lactogenic activity in rodents and immunochemical cross-reactivity with human growth hormone.

The mature placenta is shaped like an oval disc of about 500-600 g. It develops from the trophectoderm of the implanting blastocyst as it invades the endometrium. Differentiation of the placenta leads to the formation of villi structures composed of an outer layer of multinucleate syncytiotrophoblast cells formed by the fusion of underlying mononuclear cytotrophoblasts. These cells encapsulate a central core of mesenchymal cells, macrophages, and capillary epithelium.

hPL can be detected by immunofluorescence in the syncytiotrophoblast five to ten days after implantation of the fertilized ovum (4,5) or 12-17 days postfertilization. Hoshina *et al.* (6) used *in situ* hybridization to localize hPL mRNA exclusively to the syncytial layer suggesting synthesis of hPL production does not begin until formation of fully differentiated syncytiotrophoblast. Furthermore, *in situ* hybridization studies showed that the concentration of hPL mRNA in individual syncytiotrophoblast cells remains constant throughout pregnancy (6) suggesting the increase in the total amount of hPL mRNA in the placenta between first and third-trimester results from an increase in the number of

syncytiotrophoblasts in the placenta. This is in agreement with data showing the proportion of syncytial cells in the trophoblast (6-8), and the mass of the trophoblasts increase during pregnancy (8). Therefore, the increase in hPL synthesis by the placenta during pregnancy is at least partly due to an increase in the number of hPL-producing syncytiotrophoblasts.

By the third week post-conception, hPL can be detected by radioimmunassay in the maternal circulation. Peripheral serum levels of hPL then rise throughout pregnancy (9). During the third trimester hPL production can reach 1-3 grams per day (10), accounting for 10% of placental protein production and 5% of total RNA in the placenta (11, 12). At these levels hPL is the most abundant peptide hormoe produced in primates. The concentration of hPL in the maternal blood at term ranges between 5 and 15 μg/ml (8, 13, 14). hPL is present in the fetal blood but its concentration at term is 300-1000-fold lower than that of the maternal blood (13, 15, 16-18). The half-life of hPL in the maternal serum is 10-30 minutes (10, 13, 15, 19, 20) which accounts for its rapid disappearance from maternal serum after delivery.

Physiological Significance of hPL

Although much is known of the structure of hPL, the levels of hormone present throughout pregnancy, and the tissues where hPL is found, the role of placental lactogen in regard to fetal growth and metabolism was until lately poorly understood (Fig. 2). Because hPL is structurally very similar to human growth hormone (hGH),

several studies were undertaken to determine if hPL acted as a growth hormone for the fetus. Earlier bioassays using postnatal tissues and lower primate species showed hPL to have 1% of the growth promoting activity of hGH (21). More recent studies however, demonstrate that hPL has a direct somatotropic and metabolic effect in isolated human fetal tissues. Although the release of hPL is heavily biased towards the maternal circulation, the concentration of hPL in the plasma from human fetuses at 12-20 weeks gestation ranges from 4-500 ng/ml (22) and values of 20-200 ng/ml have been found in the cord blood of the newborn infant (23), suggesting a possible anabolic role in the fetus. Hill and colleagues in a series of publications were the first to make an in depth investigation into the actions of hPL upon fetal growth. They found that hPL, but not hGH, stimulates amino acid transport, [^{3}H]-thymidine incorporation, mitogenesis, as well as somatomedin-C (SM-C) and insulin like growth factor I (IGF-I) release in cultured fibroblasts and myoblasts from human fetuses at 13-19 weeks gestation (24, 25). In *in vitro* studies, hPL was found to bind to fetal tissues at hPL concentrations comparable to those found in fetal plasma, and direct anabolic effects of hPL have been found at concentrations similar to those required for binding to fetal membranes (25). Additional evidence suggests distinct hPL receptors are found in human fetal skeletal muscle, whereas there was a deficiency of fetal muscle hGH receptors (22). Evidence was also obtained suggesting unique hPL and hGH receptors are present

in fetal liver. hPL and hGH both stimulated growth of fetal hepatocytes and SM-C/IGF-I release from fetal liver. It is thought that hPL stimulates DNA synthesis in fetal connective tissue and liver indirectly through the paracrine release of the growth potentiators SM-C/IGF-I (22, 24, 25). The relationship, if any, among the hPL concentration in the fetus, the number of fetal hPL receptors and the rate of fetal growth has not been established. However, there is evidence to suggest that the weight of the fetus in early and middle gestation correlates positively with the hPL-binding capacity of the fetal liver and to a lesser extent with the fetal plasma hPL concentration (22).

Aside from its possible function as a direct acting, growth promoting hormone, substantial evidence suggests hPL influences fetal growth in an indirect manner by altering maternal metabolism. The major role of hPL in human pregnancy is believed to be mediated by its action as an insulin antagonist (4, 26). Carbohydrate tolerance was reduced in diabetics by hPL treatment (27, 28) It is believed that this action of hPL may be responsible for the development of diabetic ketoacidosis in pregnant women. hPL has also been shown to increase the insulin response to a glucose load, (19, 20, 27), perhaps as a result of an increase in insulin resistance (29).

The effect of hPL which has been most studied is its role in regulating lipolysis. Some evidence suggests that hPL is indirectly lipolytic (20, 30). Adipose tissue explants and adipocytes, stimulated by hPL, release glycerol and/or

nonesterified fatty acids (31). hPL also stimulates glucose uptake, glucose oxidation to CO_2 and glucose incorporation into glycogen, glycerol, and fatty acids in adipocytes (32, 33). This suggests a model for hPL action on adipose tissue that includes hPL-mediated increases in the basal rate of lipolysis as well as increases in glucose uptake and utilization. These actions could be physiologically significant since in the fasted state the increase in the rate of lipolysis provides free fatty acids that can be utilized as a source of energy by the mother. This spares the required glucose for use by the fetus. In the fed state, when maternal blood glucose concentration is high, the increase in glucose uptake and utilization by adipose tissue ensures that energy stores, in the form of triglyceride, will be available during subsequent periods of fasting (34). There is support for this theory as a study of women fasting for 84-90 hours during the second trimester of pregnancy reported a 30-40% increase in hPL production (35). In a similar study, fasting in pregnant women caused a much greater mobilization of free fatty acids, in the form of ketoacids, than in non-pregnant controls (36). The ability to produce PL may, therefore, be a selective advantage to foraging animals where there is the possibility of starvation, because PL causes more efficient use of energy stores and higher probability of offspring survival.

Placental lactogen received its name due to its lactogenic activity *in vivo* when administered to rabbits and pigeons (3). There

is, however, no evidence for lactogenic activity of hPL in humans *in vivo*, although there is evidence of the presence of mammotropic activity during pregnancy. hPL has been reported to stimulate DNA synthesis in epithelial cells of benign human breast tumors *in vitro* (37) and in human breast tumors maintained in athymic mice (38). It has also been reported to stimulate growth of ductal epithelium in human mammary explants obtained during lactation (39). Thus, the role of hPL in human mammary gland may be to stimulate cell proliferation, rather than milk secretion.

Evolution of The hPL/hGH Gene Cluster

On the basis of amino acid sequence homology among hGH, hPL, and human prolactin (hPRL), it was proposed that the hormones evolved from a common precursor (40). Whereas hGH and hPL share identical amino acids at 167 of 191 positions for an 87% amino acid homology, hGH and hPL are only about 35% homologous to hPRL at the amino acid level (41, 42). At the nucleotide level hGH and hPL are again very similar as mRNAs from these genes are 93.5% homologous. The hPRL mRNA has 42 and 41% identity with those of hGH and hPL, respectively (43). It is thought that GH/PL and PRL diverged approximately 350 million years ago (44, 45) when a primordial gene was duplicated giving rise to two separate precursors, one for the hGH and hPL genes and the other for the hPRL gene. The hGH/hPL and hPRL precursor genes then segregated onto two different chromosomes, since the hPRL gene is located on chromosome 6 (46) and the

hPL and hGH genes are found on chromosome 17 (47). Further evidence for the evolutionary relatedness of the hPL and hGH is that all the hPL and hGH genes are structually similar having 5 exons and 4 introns (12, 48, 49) and comparison of nucleotide sequences between any of the genes shows they share 91-99% sequence identity throughout, including 500 bp upstream of the genes (43, 44, 49, 50). It is believed that PL arose after the divergence of rodents and primates 80 million years ago (51). Evidence for this theory comes from DNA sequence data suggesting that rat PL (rPL) is derived from rPRL, as rPL is more homologous with rPRL and hPRL (52% and 51%) than rGH, hGH, or hPL (34% for all genes) (52). Therefore, duplication of the hGH/hPL gene to give rise to the hPL gene must have occurred after the separation of the main orders of mammals, during the evolution of primates (53). It is thought that the genes of the hPL/hGH gene cluster arose from their common precursor within the last 10-15 million years due to recombination events involving moderately repeated sequences (44, 54). Of the 66 kb in the hPL/hGH gene cluster, 21% of the sequence consists of 48 *Alu* repeat sequences (54). The abundance of highly homologous DNA sequences may predispose this locus to chromosomal misalignment, generating unequal recombination events (54). It was proposed that the hPL/hGH locus evolved in three steps (Fig. 3) involving large sequence duplications which preferentially begin and end with *Alu* elements (43, 54). The first event is thought to have been a duplication of a single GH/PL gene to a two-gene locus

composed of ancestral GH and PL genes. An additional duplication of this two-gene locus generated a four-gene arrangement of the ancestral hGH-N, hPL_4/hPL_1, hGH-V and hPL_3 genes. In the final step, the ancestral hPL_4/hPL_1 gene duplicated about 5 million years ago to generate the modern hPL_4 and hPL_1 genes.

Because the hPL_4 and hPL_1 genes were the last to be generated they should be the most homologous genes of the cluster. This is not the case as hPL_4 and hPL_3 are more closely related in terms of sequence homology (2.7% divergence) than the hPL_1 and hPL_4 genes (6.7% divergence) (43). However, downstream from the fourth intron the hPL_1 gene becomes more similar to the hPL_4 gene. This supports the hypothesis that hPL_4 arose from duplication of the hPL_1 gene but that a recent gene conversion event with a breakpoint in the fourth intron caused the hPL_4 gene to be more similar to the hPL_3 gene.

hPL_3 (also referred to as hCS-B or hCS-2) and hPL_4 (also refered to as hCS-A or hCS-1) share 98% sequence identity and encode identical mature proteins, but have one amino acid difference in the signal peptide. hPL_3 contains an alanine at the third position from the amino terminus of the precursor peptide, whereas hPL_4 has a proline at this position (2). The other hPL gene, hPL_1 (also referred to as hCS-L or hCS-5), was found to be a pseudogene, as no mature mRNA is produced from this gene due to a G to A transversion in the 5' splice site of the second intron (43, 54). In contrast, both hGH genes produce viable proteins. Alternative splicing of the primary hGH-N (also denoted hGH_1) gene transcript (48) generates two mRNA species that encode the major

active 22 kDa hGH protein as well as a 20 kDa variant which has 15 amino acids deleted from hGH (55, 56). The hGH-V (also called hGH_2) gene encodes a processed polypeptide differing at 13 amino acids from hGH-N. As the amino acid differences are mostly non-conservative they are thought to result in changes in the properties of the protein (11).

Gene Deletions in the Locus

The hGH/hPL gene cluster was examined in two cases in which hPL was reduced or absent in maternal serum during pregnancy. In the case of a partial deficiency of placental lactogen (57), hPL_4 was deleted in the maternal allele while the paternal allele lacked the hPL_4, hGH_2, and hPL_5 genes. The level of hPL in the maternal serum was one-fourth of the normal value, suggesting a direct relationship between gene dosage and hPL concentration in the maternal serum. Expression from the residual hPL_5 gene did not appear to compensate for loss of the other hPL genes. A direct relationship between lower hPL protein levels and decreased hPL mRNA levels was demonstrated in another case of partial hPL deficiency (58). Complete deficiency of hPL is a rare condition. Only three cases have been reported where using radioimmunoassay, no hPL could be detected during pregnancy (59-61). In the case that has been investigated in detail, a homozygous deletion of the hPL_4, hGH_2, and hPL_5 genes was found in the affected child while other family members were heterozygous for the deletion (62). The hPL_1 gene was present in the affected child but its expression was

not assayed. A novel hGH-hPL hybrid protein was detected that might have been able to compensate for the absence of hPL (63). It is not known whether this peptide is biologically active or if it is present in other cases where there is no deletion of hPL genes. However, it is conceivable that the chimeric hPL/hGH peptide could assume hPL-like roles in the mother and fetus, sustaining normal growth and development during pregnancies complicated by a deficiency of hPL production (25) Therefore at this time, the absolute necessity of a protein having hPL activity during pregnancy is still unknown.

Tissue Specific Expression of hPL and hGH Genes

Though the two genes evolved from a common ancestor and are closely linked, the hGH and hPL genes are expressed predominantly in two separate tissues. Growth hormone is secreted by somatotrophs of the anterior pituitary, whereas placental lactogen is produced by syncytiotrophoblasts in the placenta (2,6,64). In an effort to determine the levels of mRNA production of the individual genes in the cluster, Chen *et al.* (54) estimated the extent to which each gene is transcribed by the percentage of recombinant bacteriophage found in pituitary and placental cDNA libraries that hybridize to probes corresponding to each gene. In this study it was found that hGH-N accounts for 3% of the mRNA produced in the pituitary, whereas hGH-V is not expressed in the pituitary, but comprises <0.001% of mRNA in the placenta. This

hGH-V placental expression may be explained by novel recombination events during evolution of the gene cluster that placed putative hPL transcriptional control regions 5' to the hGH-V gene (54). hPL_4 was found to be the most abundantly expressed hPL gene by a 6:1 ratio accounting for 3% of placental mRNA with hPL_3 comprising an additional 0.5% of the mRNA. Studies by Barrera-Saldaña *et al.* (2) determined placental levels of hPL_3 and hPL_4 more directly. Though highly homologous, hPL_4 and hPL_3 could be differentiated by an additional *Pvu*II restriction site present in hPL_4 and a 4 bp insertion within the hPL_3 gene. These differences were exploited in restriction enzyme digests of placental cDNA clones as well as primer extension and S1 nuclease digestion of hPL cDNA-placental poly(A+) RNA hybrids. All three methods showed the ratio of hPL_4 to hPL_3 mRNA was 3:2. As these experiments reflected RNA levels from a single term placenta, hPL RNA from a further ten placentae were subjected to S1 nuclease analysis with the determination that there was wide variability in the ratio of hPL_4:hPL_3 mRNA (S.L.F. and G.F.S., unpublished data).

Regulation of hPL_3 and hPL_4 expression could be explained by sequences in the promoter that regulate transcriptional initiation or differences in the transcribed sequence that could contribute to mRNA stability. The latter theory was tested by placing each structural gene under the control of the SV40 enhancer and metallothionein promoter and transfecting COS-7 cells. Slightly more protein was produced from the hPL_3 gene construct than the

hPL_4 constuct (65). To determine the cause of such heterogeneity, the first two exons of the hPL and hGH genes were exchanged. Five-fold chimeric protein and eight-fold less mRNA was present when the first two exons of the hPL_4 were present than when the first two exons were derived from hPL_5 (Fig. 4). Protein levels resulting from the first two exons of hGH and the last three exons of hPL_4 were comparable to intact hGH, suggesting that only the first two exons contribute to differential expression. The greater stabilty of hPL_5 versus hPL_4 mRNA counters the greater expression hPL_4 mRNA *in vivo* and therefore may contribute to the variable levels of hPL_5 and hPL_4 mRNA in placentae.

The hPL_1 transcript was not detected in the original cDNA cloning (2) and lack of expression was attributed to a mutant RNA splicing site (43). Surprisingly, the hPL_1 transcript was detected by Chen *et al.* (54) in a placental cDNA library at an abundance of 0.01% using oligonucleotides specific for hPL_1, however, nine of ten cDNA clones analyzed were derived from incompletely processed hnRNAs and the other clone was found to have used an alternative splice site to overcome the mutation in the second intron splice donor site. Therefore, it is still uncertain whether hPL_1 can produce a viable protein *in vivo*. In order to determine if the aberrant splice site was wholely responsible for the lack of hPL_1 expression, a region of the hPL_1 cDNA encoding the second exon/intron boundary was exchanged with the functional equivalent region of hPL_5 (65). No protein or mRNA was produced from the

mutated hPL_3 gene or the "repaired" hPL_1 gene. The lack of function of the "repaired" hPL_1 gene is not yet understood but suggests other nucleotide differences must contribute to the lack of hPL_1 expression.

Regulation of hPL and hGH Gene Expression

The similarity of the hPL and hGH genes at the nucleotide level is striking as the hGH-N, hPL_3, and hPL_4 genes have 92.5% sequence identity through their exons, with the 464 bp 5' to the initiation site having 93.8% homology (50). The highly homologous nature of the hPL and hGH genes and 5' flanking regions suggest that very precise mechanisms regulate their tissue specific expression.

Understanding the regulation of hPL gene transcription has been facilitated by studies of hGH-N gene regulation. The hGH-N gene has been well characterized in terms of DNA sequences and transcription factors that regulate expression. It is known that 5' flanking sequences within 289 bp of the cap site mediate hGH tissue specific expression and that binding of the pituitary specific factor GHF-1 in this region permits transcription of hGH in the pituitary (66-68). GHF-1 is a 33 kD polypeptide (69) known to be closely related or identical to the Pit-1 protein that binds and regulates the rat growth hormone and prolactin gene promoters (70). Analysis of cDNA clones encoding GHF-1 indicates that it is a homeobox containing protein. GHF-1 binds the hGH promoter at two sites: -55 bp to -90 bp and -106 bp to -131 bp (68-71). In

addition to GHF-1, other general transcription factors bind to the hGH promoter (71). The Sp1 transcription factor binds nucleotides -131 to -140 adjacent to the distal GHF-1 binding site. Further upstream the USF/MLTF protein binds the region -237 bp to -267 bp, adjacent to the binding site of an unknown protein at -267 bp to -290 bp. Comparison of DNase I footprints showed the pattern of protein binding for hGH-N and hPL_3 promoters is nearly identical (72). Further studies show the pituitary specific factor GHF-1 is able to bind to the hPL promoter (71); however, this may not be significant as no GHF-1 is believed to be present in the placenta (67).

Thyroid hormone can also bind to a region of the hPL promoter in a DNA-binding assay (73). When introduced into a rat pituitary cell line, thyroid hormone and dexamethasone could increase expression of a transient reporter gene containing 500 bp of the hPL promoter (74). Therefore the hPL promoter may be regulated by dexamethasone and thyroid hormone. The physiological significance of these studies is unclear since pituitary and not placental extracts or cell lines were used.

DNA Sequences in the hPL_3 gene promoter necessary for transcriptinal activity.

The initiation sites of hPL_3 and hPL_4 gene transcription have been studied by two groups (50, 75). Both studies found 82-95% of transcripts initiate 30 nucleotides (nt) downstream from a TATA sequence (and 63 nt upstream from the AUG and start of translation). An additional initiation region 23 nt upstream of

the TATA sequence and 30 nt downstream from a CATAAA sequence was found to be responsible for 5-8% of hPL transcripts. Interestingly, the hPL_1 gene promoter was shown to be functional and acted similarity to hPL_3 and hPL_4 promoters in a cell-free transcription system (75).

More recent work has concentrated on the promoter and an enhancer of the hPL_3 gene (Fig. 5). Deletion mutants of the hPL_3 promoter were analyzed for transcriptional activity following transient transfection of a placental cell line (76). Various regions of the hPL_3 promoter extending from -1200 to -77 bp were inserted in a plasmid vector adjacent to the bacterial gene for chloramphenicol acetyl transferase (CAT). The vector also contained the hPL_3 enhancer (discussed below) to increase the level of transcription. The plasmids were transfected into JEG-3 cells, a hPL-producing placental choriocarcinoma cell line (77), with the resulting CAT activity reflecting the transcriptional activity of the hPL_3 promoter. These studies (Fig. 6) indicated sequences between -142 bp and -129 bp were important for hPL_3 promoter activity since removal of this region reduced CAT activity 8-fold. Internal deletions of this region (AR, SA, Fig. 6) confirm those seen using 5' deletions. Similar results (S.L.F. and G.F.S., unpublished data) were seen using JAR cells, another human placental trophoblast cell line (78). No other region in the promoter (within 1 kb 5' of the hPL_3 gene) was found to be important for induction of transcriptional activity in placentally derived cells.

The hPL_3 promoter regulatory sequence (-142 bp to -129 bp) does not appear to regulate tissue-specific expression based on functional and binding studies. In the presence of the hPL_3 enhancer, the hPL_3 promoter (-500/+2 bp) was able to activate CAT transcription in JEG-3 cells but not in HepG2 cells, a human liver cell line. However, paired with the SV40 enhancer, the hPL_3 promoter directed gene expression in a non-tissue specific manner as transfected JEG-3 and HepG2 cells produced similar levels of CAT activity. In these studies transcription in JEG-3 and HepG2 cells not only required the appropriate enhancer but also the hPL_3 promoter regulatory region (-142 bp to -129 bp). Without this sequence, only background levels of CAT activity were observed. This suggested that the hPL_3 enhancer was responsible for tissue-specific gene expression whereas the hPL_3 promoter regulatory sequence (-142 bp to -129 bp) was required for basal expression. While upstream sequences within 1200 bp of the hPL_3 gene did not direct transcription in a tissue-specific manner, there may be additional tissue-specific sequences further upstream. All placentally expressed members of the *hPL/hGH* gene family contain a 1 kb region of high homology located 2-3 kb 5' to the start of transcription (54) but the functional significance of this region remains to be determined.

The hPL_3 promoter regulatory sequence (-142 bp to -129 bp) binds specifically nuclear proteins from JEG-3, HepG2, and HeLa cells as shown by gel shift assay (76), again suggesting that this sequence is important for basal gene activity but does not play a

role in tissue-specific gene expression. This region of the promoter contains the sequence GGGAGG, which is a variant of the binding site GGGCGG for the ubiquitous transcription factor Sp1 (79). This variant Sp1 binding sequence, which binds Sp1, is also present in promoters of other genes including HIV LTR (80-82) rat insulin-like growth factor II (83), and human hsp 70 (84). Gel shift assays demonstrated that the addition of DNA containing the Sp1 binding site specifically competes for proteins binding the the hPL_3 promoter regulatory sequence. Further competition studies employing a variant Sp1 site GGGGG, which is not recognized by Sp1 (85), showed that this oligomer did not compete for JEG-3 proteins binding to the hPL_3 promoter regulatory region. The binding specificity of the proteins interacting with the hPL_3 promoter regulatory region was further characterized by determining nucleotides of the hPL promoter regulatory sequence interacting with DNA-binding proteins using a methylation interference assay. The methylation interference pattern of placental nuclear proteins binding to the hPL_3 regulatory sequence was identical to that shown previously for Sp1 binding to its binding sites. These results suggest that the hPL_3 regulatory region binds Sp1 or an Sp1-like protein which is necessary for basal transcriptional activity.

Sp1 regulates transcription of many different kinds of genes (79). However it does not directly interact with RNA polymerase (86) or the transcription factor TFIID, which binds the TATA sequence (87, 88), suggesting that Sp1 requires accessory factor(s) or coactivators to activate transcription (88). One such factor

could include the protein(s) binding the tissue specific hPL_3 enhancer.

An Enhancer 3' of the hPL_3 Gene Stimulates Tissue-Specific Transcription.

In order to explain the tissue specific expression of the hPL_3 genes Rogers *et al.* (89, 90) conducted a search of the entire hPL/hGH gene cluster for the presence of transcriptional enhancers. A series of *Eco*R1 fragments covering nearly the entire 66 kb gene cluster including the five genes and their flanking regions were tested for enhancer activity. Only one *Eco*R1 fragment, was found to have enhancer activity in JEG-3 cells. This 3.9 kb fragment was located 3' to the hPL_3 gene at the distal end of the hPL/hGH gene cluster (Fig. 7). This fragment fulfilled all the required definitions of an enhancer as it was active either 5' or 3' of a gene and in either orientation and was able to activate a heterologous promoter.

Further analysis of the hPL enhancer localized enhancer activity to a 1 kb *Acc*I - *Sac*I restriction fragment located 2 kb 3' of the hPL_3 gene. The 1 kb enhancer was shown to have great tissue preference in its action as the enhancer was 20-fold more active in human trophoblast JEG-3 cells than the rat pituitary cell line 18-54-SF (89). Therefore, the hPL enhancer is likely to be the element responsible for the tissue specific-expression of the hPL_3 gene.

Further transient transfection experiments employing JEG-3 and JAR cell lines allowed localization of the hPL enhancer to a 138 bp region located between nucleotides 103-241 of the 1 kb *Acc*I - *Sac*I restriction fragment (Fig 8) (91). The 138 bp hPL enhancer when linked 3' to the CAT gene increased CAT expression 25 to 30-fold over that due to the SV40 promoter alone. The 138 bp region also contained all information necessary to impart tissue-specific gene expression as plasmid constructs containing this region are 20 to 40-fold more active in JEG-3 cells than non-hPL producing human HepG2 liver, U-373 MG glioblastoma and, HeLa epithelial cell lines. Additional studies support the idea that the hPL enhancer may be the major determinant of tissue-specific expression of the hPL_3 gene. As stated previously, Fitzpatrick *et al.* (76) have shown that hPL_3 promoter fragments extending to -496 bp upstream of the gene had similar activities in JEG-3 and HepG2 cells. Therefore, the hPL enhancer is the major positive tissue specific regulator of hPL_3 gene transcription.

Enhancer-Nuclear Protein Interactions

A 210 bp fragment (1-210) containing all the information necessary for tissue-specific enhancer function was found to specfically interact with nuclear proteins from placenta tissue, JEG-3 and JAR cells. Band retardation assays showed that up to three specific protein-enhancer complexes are formed with protein extracts from placental lactogen-producing cells. These complexes

are identical for all the extracts from hPL-producing cells. However, protein-DNA complexes of different electrophoretic mobilities and affinities were formed following the incubation of HeLa and HepG2 nuclear proteins with the 1 - 210 bp enhancer fragment.

DNase I protection experiments showed that proteins from both placental and HeLa cell nuclear extracts protect a 22- bp region (115 - 137) of the hPL enhancer encompassing a TEF-1 motif (TGGAATGTG) located at positions 126 - 133 (Fig. 9). The TEF-1 motif was first identified by Xiao *et al.* (92) in studies of the SV40 enhancer and has been shown to bind a 53 kDa protein called TEF-1 (93). Binding of this protein to the TEF-1 motif correlates with *in vivo* SV40 enhancer activity (92). TEF-1 protein is found in cell types (HeLa and undifferentiated and differentiated F9 embryonal carcinoma) other than those that produce hPL. However, TEF-1 shows some tissue specificity, as no TEF-1 activity is found in lymphoid cell lines (92, 93). The TEF-1 protein binds cooperatively to tandem repeats of the TEF-1 motif or to closely linked *Sph*I (AAGCATGCA) or *Sph*II (AAGTATGCA) motifs. When present in tandem repeats or when associated with Sph motifs, TEF-1 is a strong transcriptional activator.

The region of DNase I protection (115-137) seen over the TEF-1 motif using the hPL enhancer is similar to that reported in studies of the TEF-1 motif in the SV40 enhancer (93). Though these two enhancers have no sequence similarity except for the nine

nucleotide TEF-1 site, a 22-nucleotide region of the hPL enhancer encompassing the TEF-1 motif is protected by placental and HeLa extracts, whereas a 21-nucleotide region of the SV40 enhancer is protected by HeLa nuclear extracts. This suggests a protein similar to TEF-1 binds the hPL enhancer. However, band retardation experiments using the hPL enhancer showed that proteins from HeLa and placental extracts have different affinities for the 1-210 bp enhancer fragment and the protein-DNA complexes formed due to these proteins have dissimilar mobilities in polyacrylamide gels (91). Also, placental and HeLa nuclear proteins cause different patterns of DNase I hypersensitivity, suggesting their binding alters DNA conformation in different ways. This difference in binding characteristics of placental and HeLa enhancer binding proteins may reflect different protein modifications, a family of TEF-1 like proteins with individual members expressed in specific tissues, or different regulatory proteins that bind to similar DNA recognition sites.

Regions 3' of the hPL_1 and hPL_4 Genes are Homologous to the hPL_3 Gene Enhancer

Inspection of the hPL/hGH gene cluster DNA sequence revealed the presence of two other putative enhancers located 3' to the hPL_1 and hPL_4 genes (91). The enhancers are located in the same relative location as the hPL_3 enhancer, approximately 2.2 kb 3' to their respective genes. These regions are 95% homologous with the 1022 bp hPL_3 enhancer and the region shown to interact with placental nuclear protein(s) by DNase I footprinting is exactly

duplicated in both the hPL_1 and hPL_4 flanking regions (Fig. 10). No homology to the hPL_3 enhancer was detected anywhere else in the 66 kb hPL/hGH gene cluster including hGH-N or hGH-V flanking regions.

An explanation for the limitation of enhancers to the 3' flanking regions of the hPL genes has been proposed (42). It is postulated that the 3' breakpoint of the original duplication event leading to the precursor hPL and hGH genes occurred within an *Alu* repeat about 100 bp downstream from the hPL-hGH ancestral gene. It is thought that the duplication event most likely involved a homologous, unequal crossover between this *Alu* repeat and another found 8 kb upstream of the hGH_1 gene (see Figure 3). The major significance of the initial duplication event is that it would cause the 3' regions close to the hPL and hGH genes to be unrelated. This would account for the presence of a placenta-specific regulatory element 3' of hPL but not hGH genes. Also the presence of an enhancer 3' of the hPL_4 gene would explain why hPL_4 mRNA was found to be at least as abundant or more abundant than hPL_3 mRNA (2, 54) while lack of enhancer homology near hGH genes correlates with low placental hGH expression.

FIGURE LEGENDS

Fig. 1. The primary amino acid structure of human placental lactogen. The two disulfide bridges are marked by heavy shading. Reproduced with permission from Li, 1972 (1).

Fig. 2. Physiological role of human placental lactogen during pregnancy. The current model of the functional role of hPL in maternal metabolism is, by its lipolytic and insulin antagonist activity, to increase preferentially glucose availability for the fetus. (-) = inhibitory effect. Reproduced with permission from Yen, 1989 (94).

Fig. 3. Schematic diagram of the evolution of the hGH/hPL gene family redrawn with permission from Hirt *et al.*, 1987 (43). Gene sequences are indicated by stippled boxes, *Alu* sequences noted by solid boxes and partial *Alu* sequences noted by open boxes. The latter indicate differences between the 3' ends of the hPL and hGH genes. The present day gene family is believed to have originated from a single ancestral gene by three duplication and nonreciprocal crossover events ((X)).

Fig. 4. Diversity in size and abundance of protein expression products from hPL and recombined hPL/hGH genes. Reproduced with permission from Reséndez-Pérez, 1990 (65). A. Map of the hybrid

genes which are composed of hPL-3 (solid) or hPL-4 (hatched) and hGH (open) gene portions. **B.** Proteins produced from COS-7 cells transfected with hPL/hGH hybrid genes. As a negative control lane (-), COS-7 cells were transfected with a vector lacking hPL and hGH gene sequences. Intact hGH genes produce 20 kD and 22 kD proteins while the hPL protein is 25 kD. **C.** Slot Blot analysis of hPL-3 and hPL-4 RNA expression. Total cellular RNA was obtained from COS-7 cells transfected with a vector containing either the hPL-3 or hPL-4 genes. RNA was applied to each slot of a slot blot apparatus and the filter hybridized to an hPL cDNA probe and a DHFR cDNA probe.

Fig. 5. Restriction map of the hPL_3 gene and flanking region. Promoter deletion mutants were constructed from the 500-bp *Eco*R1 (-497 bp)/*Bam*HI (+2 bp) sequence. An enhancer is located 2.2 kb 3' to the gene within the *Acc*I-*Sac*I restriction fragment. Open boxes, Human PL_3 exons. Restriction enzyme cleavage site abbreviations are as follows: AI, *Ava*I; AII, *Ava*II; Ac, *Acc*I; Au, *Alu*I; B, *Bam*HI; D, *Dde*I; E, *Eco*RI; H, *Hinf*I; P, *Pvu*II; Ps, *Pst*I; S, *Sac*I; Sa, *Sau*3A; St, *Stu*I; X, *Xba*I. Reproduced with permission from Fitzpatrick, 1990 (76).

Fig. 6. Transient expression of hPL_3 CAT deletion mutants in JEG-3 cells. **(A)** Deletion mutants of the hPl_3 promoter with varying amounts of 5' sequence (relative to the start of transcription)

were ligated 5' to a promoterless bacterial gene CAT. Three internal deletion mutants, -232 AR (Δ-129/-77), -232 MR (Δ-142/-77), -390 SA (Δ-152/-129), were also constructed. Horizontal bars indicate DNA sequences included in each construct. The hPL_3-CAT DNA was cotransfected with a reporter plasmid containing the β-gal gene into JEG-3 cells, a human placental choriocarcinoma cell line. CAT activity was expressed relative to β-gal activity as the mean ± SD. (B) JEG-3 cells were transfected with plasmids containing the hPL_3 promoter mutants described in A, and the CAT activity is shown by the autoradiograph. AR, MR, and SA refer to internal deletion mutants (A). Positive (pSV2CAT) and negative (pCAT3M; mock) controls were included. Reproduced with permission from Fitzpatrick, 1990 (76).

Fig. 7. Restriction map of the human placental lactogen and growth hormone multigene cluster. The map and gene nomenclature is a composite from maps previously published. The structural gene regions are indicated by the open blocks and the direction of transcription is from left to right. Each of the vertical bars represent an *Eco*RI restriction site. Reproduced with permission from Rogers and Saunders, 1986 (90).

Fig. 8. Activity of hPL_3 enhancer deletion mutants in JEG-3 cells. JEG-3 cells were transfected with an SV40-enhancer containing vector (pSV2CAT), enhancerless vector (pSV1ØAT), or pSV1CAT

constructs containing various deletion mutants of the 1022 bp *Acc*I-*Sac*I hPL_3 enhancer. Activity of each construct is normalized to activity of pSV2CAT = 100%. Horizontal bars refer to the region of *Acc*I-*Sac*I fragment remaining in constructs. Reproduced with permission from Walker *et al.* 1990, (91).

Fig. 9. Interaction between placental nuclear proteins and DNA sequences in the hPL_3 enhancer. A DNA fragment containing the hPL_3 enhancer was incubated with a placental nuclear extract and digested with DNAse I. The region protected from enzyme digestion is shown by the brackets.

Fig. 10. Comparison of hPL_2 and hPL_1 3' flanking regions with the hPL_3 enhancer. Slashes represent gaps in nucleotide homology. Underlined nucleotides signify the TEF-1 motif.

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Walker, W.

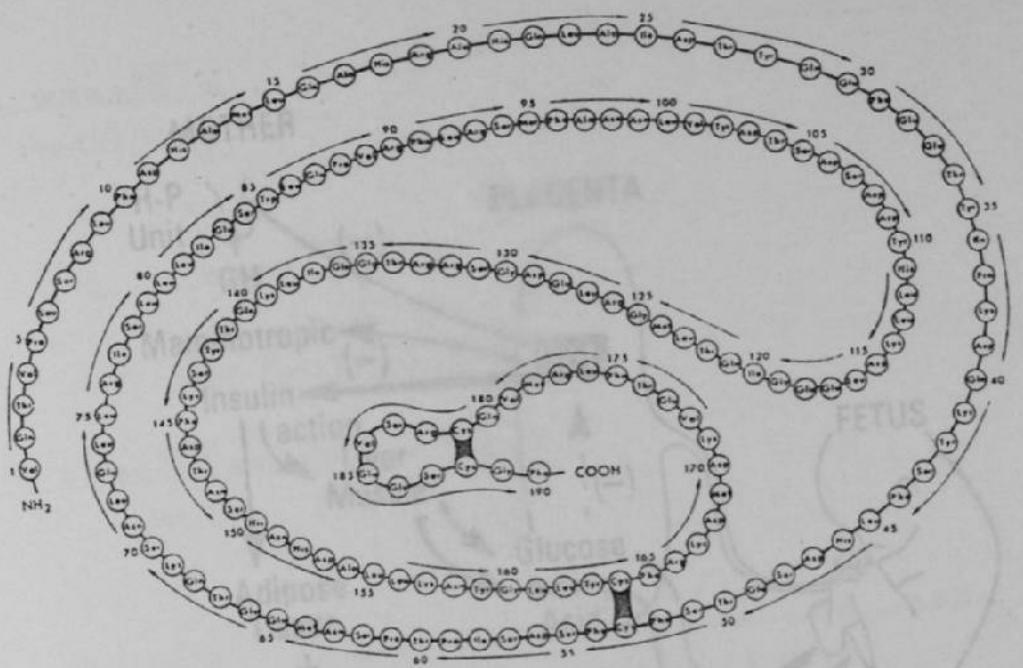


Figure 1

Walker, W.

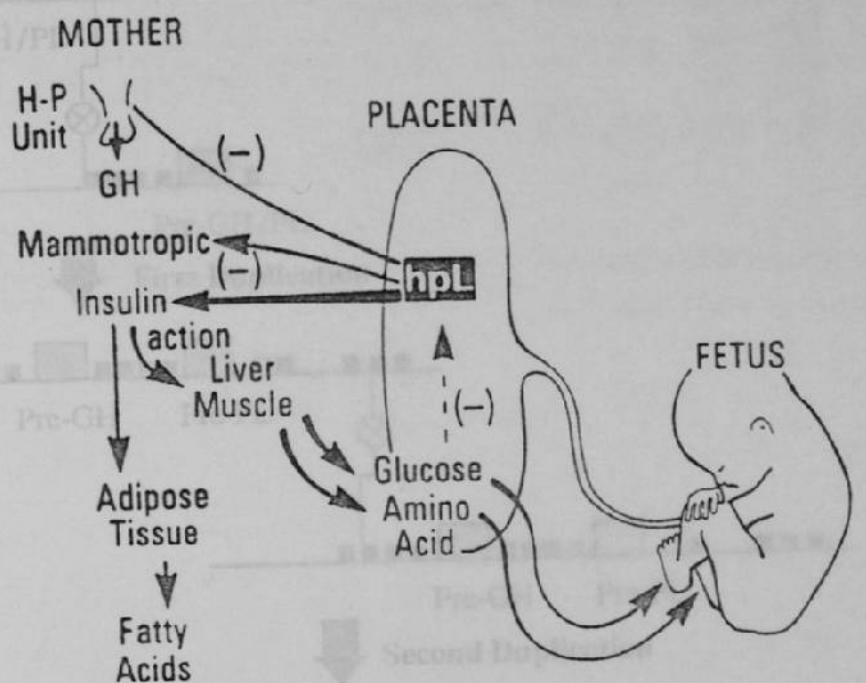
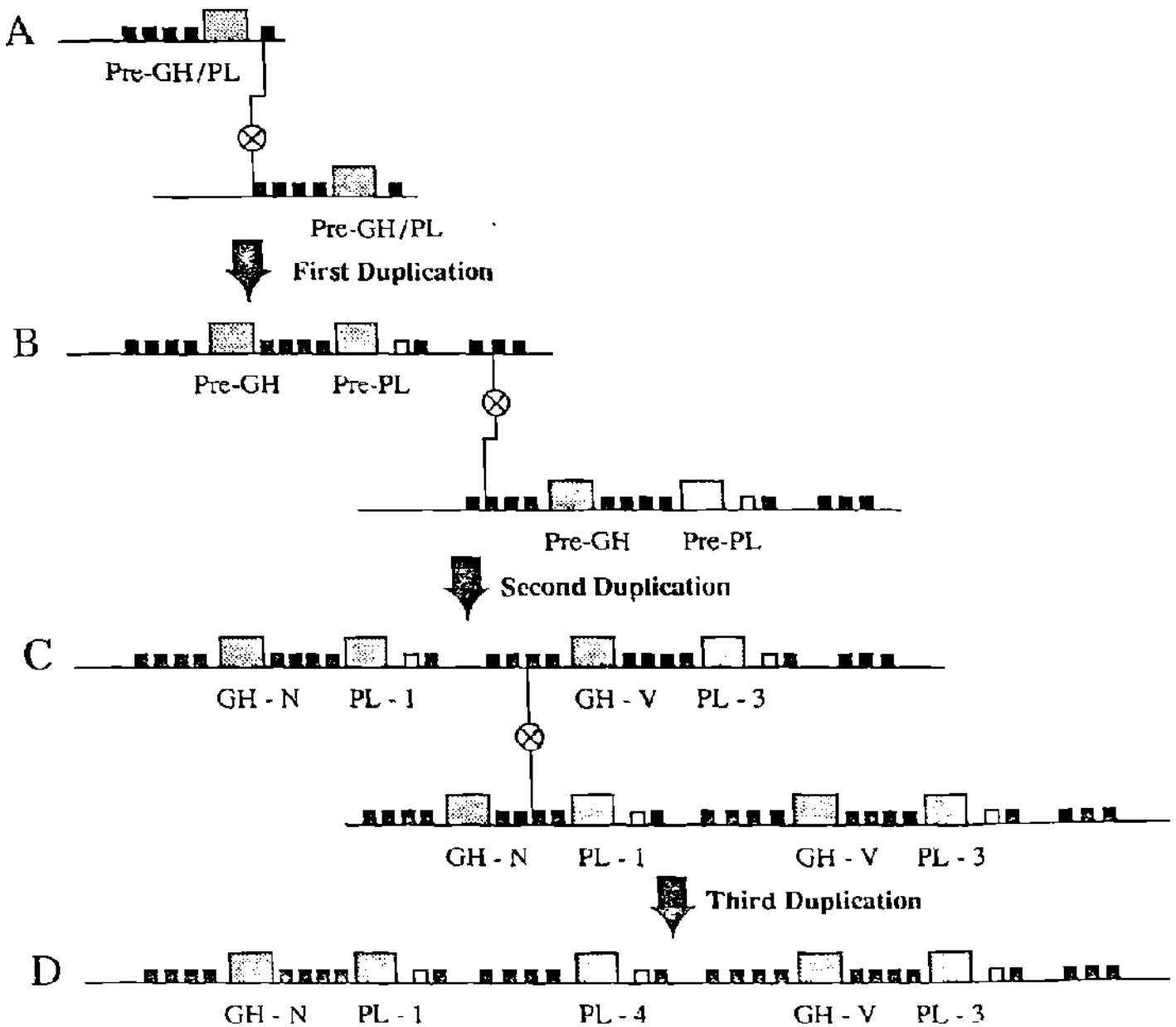


Figure 2

Evolution of the Growth Hormone / Placental Lactogen Gene Family



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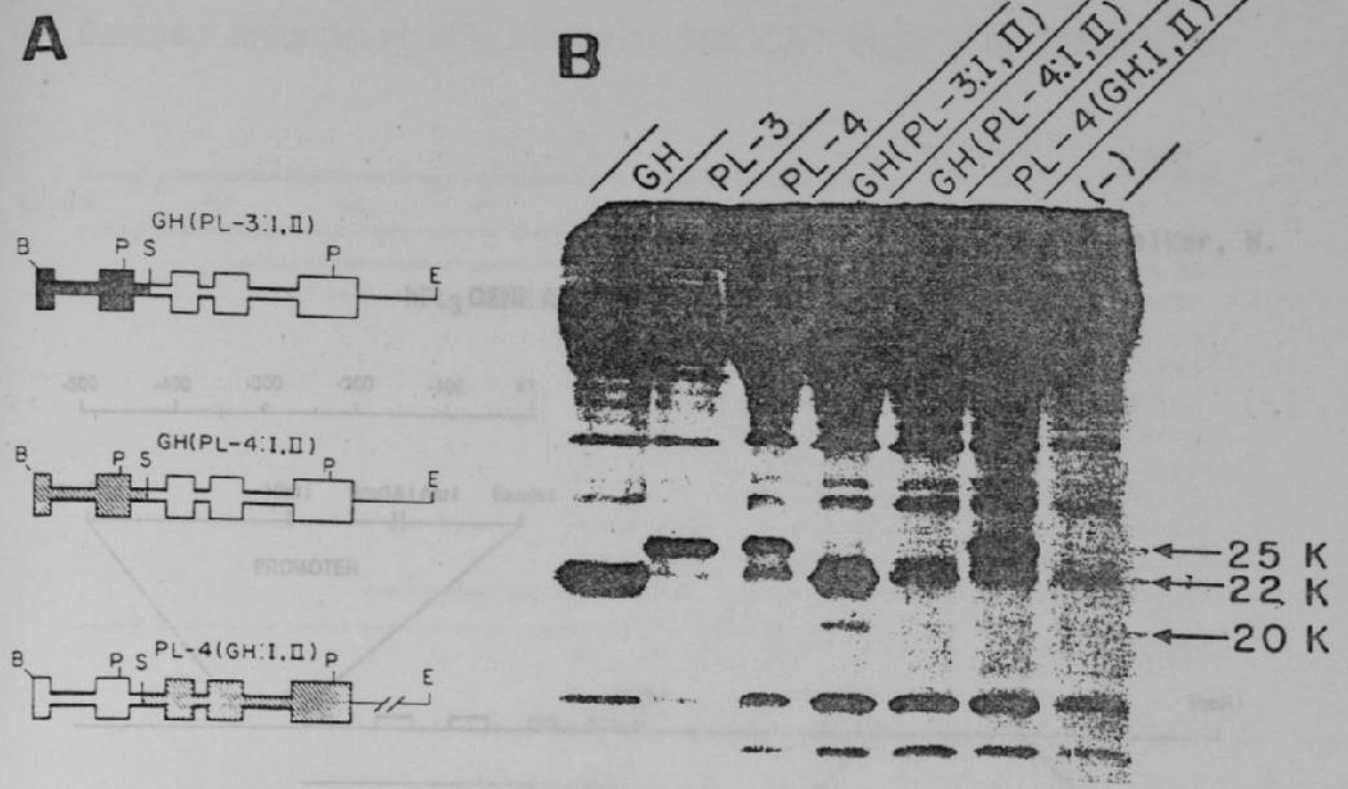

Figure 4A,B

Walker, W.

Figure 4C

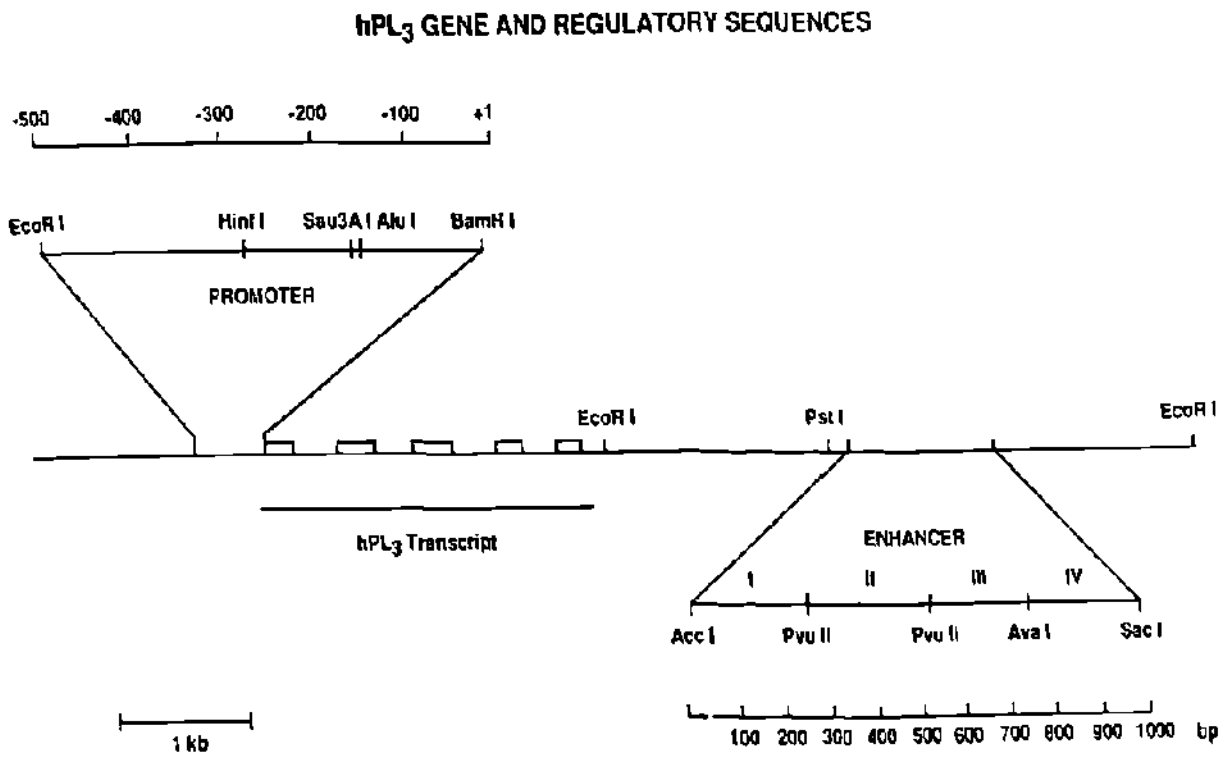
hPL3 GENE AND REGULATORY SEQUENCES
-500
-400
-300
-200
-100
+1
EcoR I
Hinf I
Sau3A I
Alu I
BamH I
PROMOTER
EcoR I
Pst I
EcoR I
hPL3 Transcript
ENHANCER
I
II
III
IV
Acc I
Pvu II
Pvu II
Ava I
Sac I
1 kb
100 200 300 400 500 600 700 800 900 1000 bp

Figure 5

Deletion mutants of hPL promoter and CAT activity in JEG-3 cells

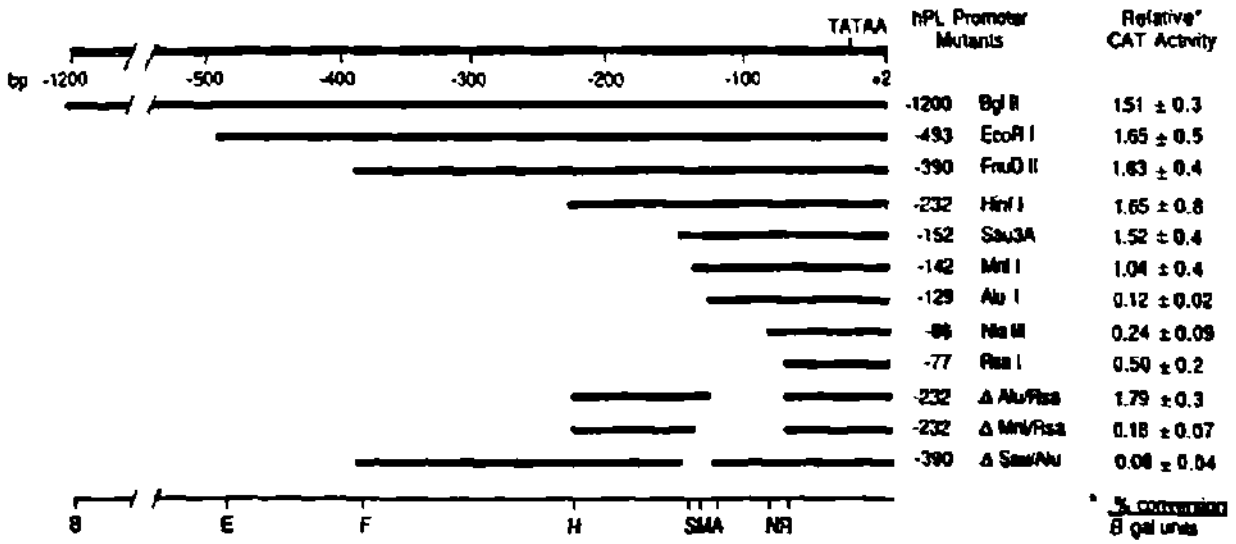

Figure 6A

Walker, W.

Activity of hPL Promoter Deletion Mutants in JEG-3 Cells

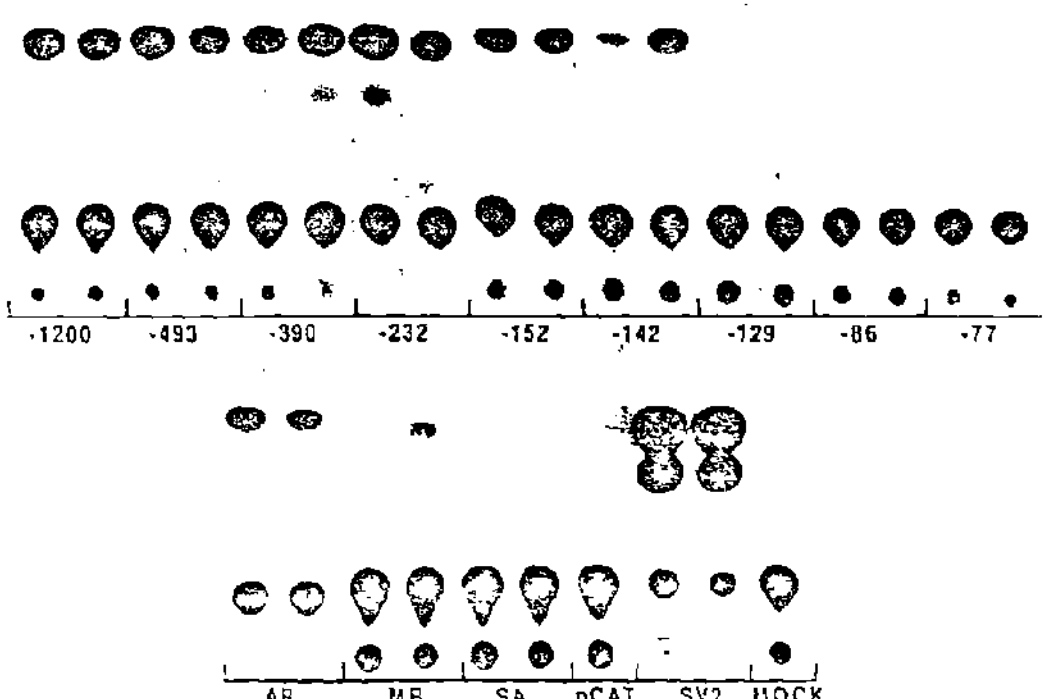

Figure 6B

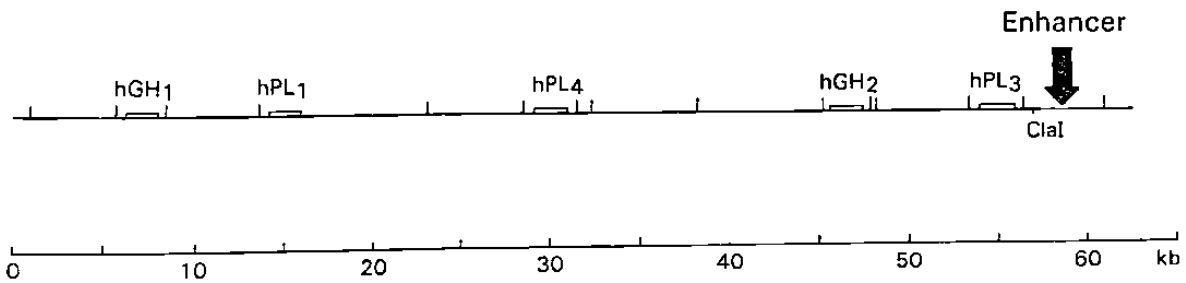

Figure 7

Summary of hPL Enhancer Deletion Mutants Activity in JEG-3 Cells

Construct	% pSV2CAT	S.D.	Ob
1) 1-1022	84	14	11
2) 1-730	45	11	7
3) 158-1022	0.7	0.1	7
4) 289-932	1.6	1.4	5
5) 1-134	2.0	1.4	6
6) 1-210 (+)	79	17	7
7) 1-210 (-)	72	9.8	6
8) 103-404	87	33	9
9) 103-241 (+)	78	24	4
10) 103-241 (-)	61	18	4
11) Δ241-506	40	16	8
12) 241-506	1.2	0.1	6
13) 210-404	2.0	1.0	6
14) pSV1CAT	1.2	1.2	6

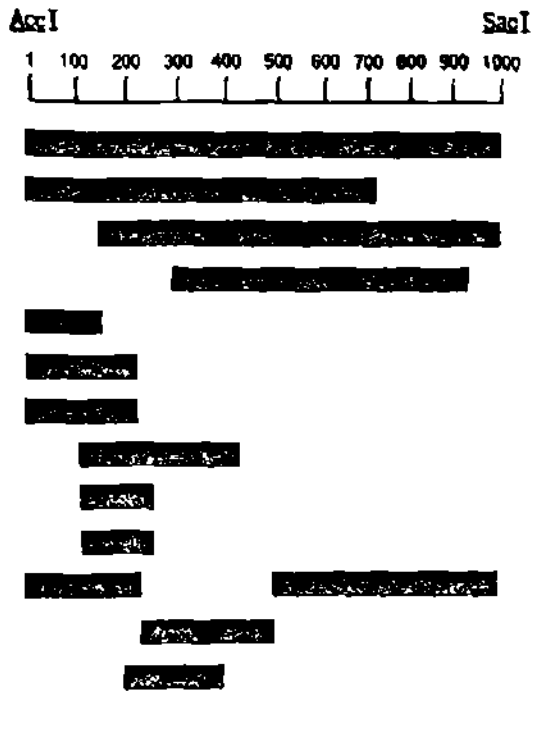

Figure 8

Summary of hPL Enhancer DNase I Protected Regions

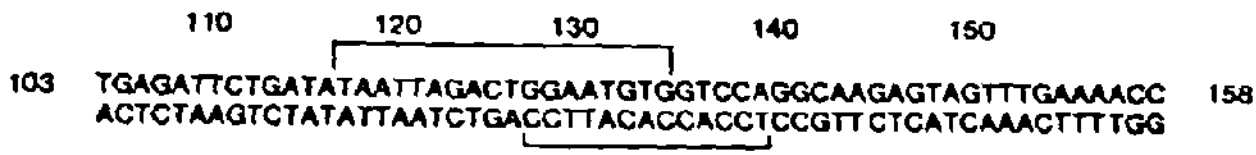

Figure 9

Homology of hPL_3, hPL_1 and, hPL_4 3' Flanking Regions

hPL_3 110 120 130 140 150

TGAGATTCTGATATGGTTAGACTGGAATGTGGTCCAGGCAAGAGTAGAGTTTGAAAAC

hPL_1

TGAGATTCTGATATGGTTAGACTGGAATGTGGTCCAGGCAAGAGTAG//TTTGAAAAC

hPL_4

TGAGATTCTGATATGGTTAGACTGGAATGTGGTCCAGGCAAGAGTA/A/TTTGAAAAC

Figure 10

ANEXO C

xpression Studies of ransfected Multigene amilies by Homologous NA Mutagenesis

BSTRACT

A valuable approach for multigene mily studies where the expression oduct of at least one gene member of e family is measurable is described. In ch cases, the effect on gene expression nucleotide sequence differences or utations occurring in other members of e family or at alleles can easily be deter ined. This is achieved by a strategy alled homologous DNA mutagenesis. It nsists of the insertion of mutated gions from homologous genes into the ntext of the gene coding for the assay ble product. Here we demonstrate the asibility of this approach using gene embers of the human growth hormone nd human placental lactogen (hGH-hPL) ultigene family.

NTRODUCTION

Advances in recombinant DNA echnology have made the task of solating genes from higher eukaryotes asier. However, many of the protein-ncoding-type genes exist in the mammalian genome as families with multiple non-allelic copies with very similar sequences. An approach to studying multigene families has been to extensively examine one member and then predict the nature of its expressed protein and those expressed by its related members. Nevertheless, proving that all members of the multigene family are expressed can be a formidable task.

Several strategies have been employed to achieve this goal. For example, if the presence of specific mRNAs on a polysome fraction can be demonstrated, then it may be suggested that translation occurs and that the genes are being expressed (7). In other cases, the use of mRNA complementary DNA (cDNA) cloning in conjunction with prokaryotic expression vectors permits the generation of antibodies. These are raised against fusion proteins expressed by the vector along with the cDNA coding protein (3). The antibodies then can be used to investigate the *in vivo* temporal and cellular parameters for the expression of these genes.

In studies of gene expression, it is ideal to be able to directly characterize the expressed protein. However, to quantitatively discriminate from at least 50 000 other proteins of the eukaryotic cell, the vector's expression control signals must be strong and, preferably, should be of wide tissue specificity. There are several vectors that fulfill these criteria and take advantage of the use of transcriptional control signals from mammalian viruses (5).

Mutations can affect expression levels in eukaryotic cells due to an aberration in any one of the many steps of the gene expression pathway: transcription, RNA processing, mRNA stability or translation (15). The effects of single base mutations in the expression of structural sequences are frequently studied but require a more selective, analytical method of assessing such changes.

Here we describe a strategy, called homologous DNA mutagenesis, to accurately evaluate the effect of small mutations occurring at pseudogenes, functional non-allelic copies or mutated alleles of multigene families. It consists of the introduction of the mutated area, with a minimal amount of flanking DNA sequences, into the structural region encoding a homologous so-called reporter gene for which antibodies or a quantitative assay are available. We used this system to examine the influence on gene expression of sequence variations found in hPL genes. In addition, we inserted a mutation occurring at a putative hPL pseudogene (1) into the context of the hGH structural gene. The convenience of using hGH as a reporter protein (13) allowed us to use a simple radioimmunoassay to quantify the effect of these nucleotide sequence changes.

MATERIALS AND METHODS

Construction of Recombinant Plasmids and Preparation of Their DNA

Restriction and other enzymes were obtained from commercial suppliers (BRL/Life Technologies, Gaithersburg, MD, and New England BioLabs, Beverly, MA) and used according to the manufacturers' instructions. The characterization of hPL genes has already been described (1). The vector described here was derived from pNUT (9), a gift from Richard Palmiter, and consisted of the larger DNA fragment resulting from *Bam*HI plus *Eco*RI digestion of this plasmid. This fragment was isolated (14) and ligated to the DNA fragments, flanked by the same sites, carrying the gene fragments of interest. Ligations and transformations were carried out as previously described (2,8). Recombinant plasmids were first isolated from 3-ml overnight cultures and then characterized by restriction analysis, followed by large-scale preparation (8). pMThGH111 (10) was also a gift from Richard Palmiter, and pSV2gpthGH (12) was a gift from Grady Saunders. pCMVhGH was constructed in our laboratory by replacing the chloramphenicol acetyltransferase (CAT) sequences in pCMVcat (4) with structural hGH gene.

Cell Culture, DNA Transfection and Detection of Transiently Expressed Proteins

COS-7 cells (a gift from Tien Kuo) were adapted to grow in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO) containing 1% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT). They were maintained at 37°C with 5% CO_2. Plasmid DNA (7.5 µg/60-mm-diameter Petri dish) was transfected by the calcium phosphate method (6). Efficiency of transfections was normalized by co-transfecting with pCMVcat and monitoring CAT activity (5).

Quantification of hGH and hPL-hGH chimeric proteins was achieved using a commercially available radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). Media from cultured cells were diluted and assayed using the protocol included with the kit and briefly summarized as follows: 0.1 ml of calibrators (ranging from 0–30 ng/ml, the latter concentration corresponding to 60 µIU/ml of the World Health Organization standard) and diluted samples were added to 12 × 75-mm tubes. Anti-hGH rabbit serum (0.1 ml) was added to the tubes and the mix-

tures were incubated for 1 h at room temperature. This antibody is known to be highly specific for hGH. It presents cross-reactivities to hPL, thyroid stimulating hormone (TSH), follicle stimulated hormone (FSH), luteinizing hormone (LH) and towards a subunit β of human chorionic gonadotropin (HCG) of less than 0.006%. To each tube was added 0.1 ml of ^{125}I-hGH and incubation continued for another hour. A second antibody (goat anti-rabbit serum) (1.0 ml) mixed with PEG in saline solution was added. After centrifugation, the supernatant was decanted, and the radioactivity remaining in the tubes was determined. Cpm values of experimental samples were used to estimate hGH concentrations using the known hGH concentrations of the calibrators as reference.

RESULTS AND DISCUSSION

Choice of Expression Vector

The radioimmunoassay values of secreted hGH from DNA transfection experiments were compared for plasmid constructs pSVgpthGH, pMThGH, pCMVhGH and pNUT. As illustrated in Figure 1, in plasmid pSVgpthGH, the hGH structural gene is under the control of natural hGH gene promoter and a copy of the simian virus 40 (SV40) enhancer. The second construct, pMThGH, has the hGH structural gene joined to the metallothionein promoter. The third construct, pCMVhGH, has the cytomegalovirus enhancer-promoter pair driving the expression of the hGH structural gene (11). Finally, pNUT uses the metallothionein promoter and SV40 enhancer to express the promoterless hGH-N gene. The level of hGH secreted into the medium of cells transfected in parallel with each of these plasmid constructs was found to be highest with pNUT (data not shown); therefore, we chose pNUT for the rest of the study.

Table 1. Transient Expression[a] of Human Growth Hormone Secreted Into the Media of Transfected COS-7 Cells

Gene	hGH Production[b]
None	0.3 ± 0.73
hGH-N	656.03 ± 126.32
GH(PL-3:I,II)	408.68 ± 69.79
GH(PL-3m:I,II)	0.26 ± 0.68
GH(PL-4:I,II)	82.94 ± 30.31

[a] Plasmids transfected consisted of the pNUT vector described in Materials and Methods, carrying as insert the gene indicated.

[b] Values, average and standard deviations, are in ng of hGH/culture dish, obtained by radioimmunoassay. Each represents data from three independent cotransfection experiments using a different set of plasmid DNA preparation and pCMVcat to compensate for variations in transfection efficiency.

Homologous DNA Mutagenesis

We chose to examine the effects of two different types of mutations on gene expression. The first being that of sequence variations among the first two exons of the hPL-3 and hPL-4 genes. The second corresponding to an important donor splice point mutation (GT→AT) found at the beginning of the second intron of the putative hPL pseudogene (hPL-1 or hPL-like gene). For the above purpose, we designed a strategy called homologous DNA mutagenesis, whereby we inserted the DNA regions of interest from the homologous genes into the context of the gene coding for hGH. A segment comprising the first 588 base pairs (bp) of the gene from the capping site to the second intron of the hGH gene was replaced by the corresponding segments from hPL-3 and hPL-4 genes. These 588-bp segments from the two active hPL genes display only eight nucleotidic changes distributed as follows: one in the 5'-untranslated region, one in the first exon (signal peptide), three in the first intron and three in the

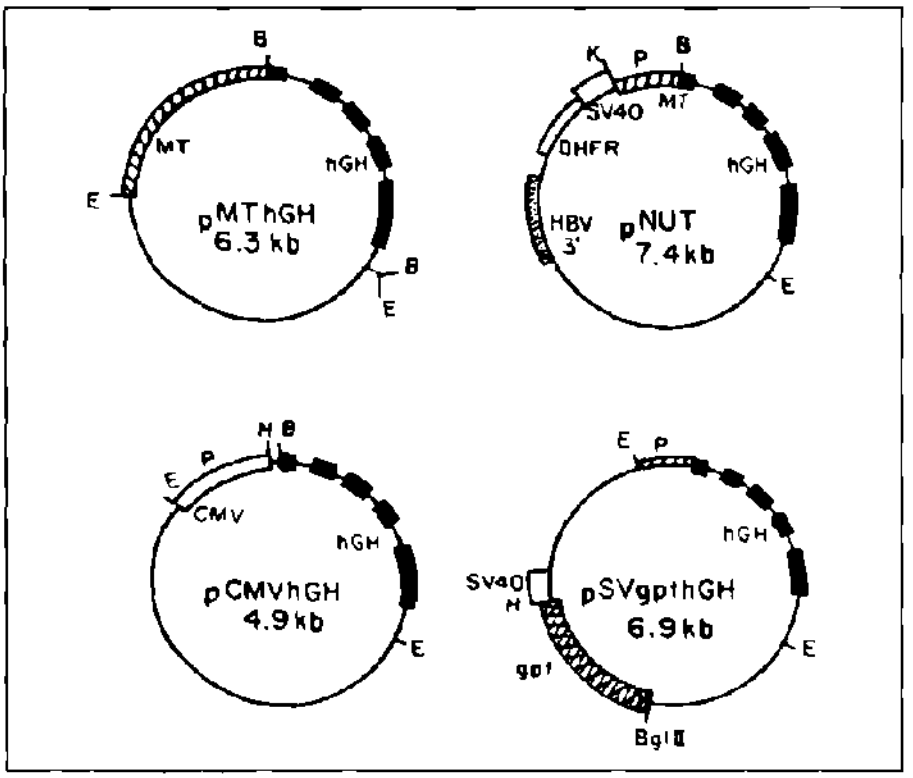

Figure 1. Structure of hGH expression vectors. With the exception of pSVgpthGH, which contains the entire hGH gene including its natural promoter, all other vectors shown here were constructed by fusing the hGH gene structural region between the *BamH*I site at the beginning of the first exon to the *Eco*RI site at the 3' end to different transcriptional control elements. In pMThGH, the expression of the hGH promoter-less gene is directed by the mouse metallothionein promoter. In pNUT, the SV40 enhancer and the mouse metallothionein promoter control the level of transcription. Finally, in pCMVhGH the transcriptional control element is the enhancer-promoter of the immediate early transcribed region of human cytomegalovirus

second intron. We also replaced this same region for sequences from the hPL-3m hybrid gene. hPL-3m ("m" means mutated) is a derivative from the active hPL-3 gene constructed in our laboratory (R. Ramírez-Solís and H.A. Barrera-Saldaña, unpublished results) carrying the point mutation at the splice site described above. Maps of these chimeric genes are shown in Figure 2.

Transient Expression Levels of the hGH Structural Genes Containing Mutations

To quantify the effects of the expression of the sequence changes introduced into the hGH structural gene after their construction, we inserted the hybrid genes into pNUT. Both the plasmids carrying the hybrid genes and control plasmids were introduced into COS-7 cultured cells. We found that the media from cells transfected with the plasmid carrying the hGH-N/hPL-3 hybrid gene [GH(PL-3: I, II)] contained hGH equivalent to 62.3% of that found with the positive control (pNUT). Values of hGH, five times lower than those obtained with the hGH-N/hPL-3 hybrid gene, were detected in the media of cells transfected with the hGH-N/hPL-4 hybrid gene [GH(PL-4: I, II)] plasmid. The hGH level in the media of cells transfected with the plasmid vector having as insert the hGH-N/hPL-3m hybrid gene [GH(PL-3m: I, II)] was negligible. These results (see Table 1) suggest that the splice site mutation at the beginning of intron II of the hPL-1 gene may be the cause of a reduced expression of this gene. In addition, they help to reveal sequence variations within the first 600 bp of the 2 active hPL genes as a possible source of difference in the hPL gene expression level.

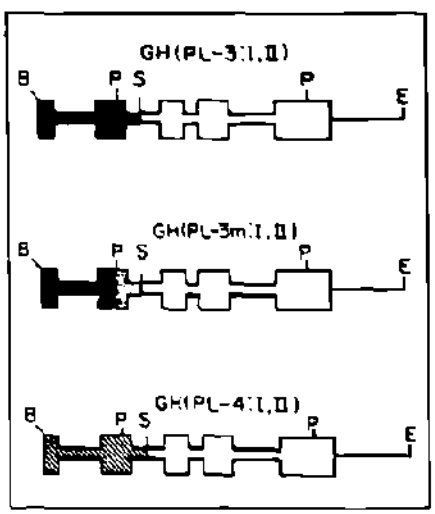

Figure 2. Genetic organization of chimeric gene constructs. *The drawings illustrate the* maps and origin of the gene fragments (2150 bp) used for the construction of the chimeric genes used. Boxes represent exons. Sites for restriction enzymes are: B = *Bam*HI, P = *Pvu*II, S = *Sac*I and E = *Eco*RI. Origins of gene fragments are in black, hPL-3; stippled, hPL-1; hatched, hPL-4; and open, hGH-N.

CONCLUSIONS

A novel aspect of our strategy is the use of hGH, a convenient extracellular reference protein (13) to quantify the effect of mutations on gene expression. It is particularly helpful when studying the expression of a gene related to the reporter gene. This is achieved by homologous DNA mutagenesis, which consists of the insertion of mutations present in members of a multigene family into the context of a homologous reporter gene. Using the gene coding for the well-known form of hGH, our approach permits a quantitative measurement of the effect of practically any mutation or nucleotide sequence change occurring in placental lactogen and growth hormone genes. Our procedure should be possible to use with any gene whose protein product can be easily detected.

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