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New vectors for the efficient expression of mammalian genes in cultured cells

(Transient expression; cytomegalovirus enhancer-promoter unit; multiple cloning site; *hGH* gene and cDNA; polyadenylation site)

Ramiro Ramírez-Solís, Diana Reséndez-Pérez, Luis Enrique Alvidrez-Quihui, Diego Enrique Rincón-Limas, Ricardo Varela-Martínez, Herminia G. Martínez-Rodríguez and Hugo A. Barrera-Saldaña

U.L.I.E.G. Departamento de Bioquímica, Facultad de Medicina, U.A.N.L., Monterrey, N.L. (México 64460)

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SUMMARY

We have constructed a new pair of plasmid vectors for the efficient expression of mammalian genes. The first of the new plasmids, pAVE1, was derived from pCMVcat [Foecking and Hofstetter, Gene 45 (1986) 101–105] by replacing the chloramphenicol acetyltransferase-encoding sequences in the latter for a multiple cloning site. Since it possesses the powerful enhancer-promoter unit of the immediate early gene of human cytomegalovirus, pAVE1 is ideal for the expression of mammalian genes. The second expression vector, pAVE2, resulted when the 3′-end flanking region from the human growth hormone-encoding gene (*hGH*) was incorporated in pAVE1. This region provides sequences for 3′-end processing and polyadenylation of primary transcripts. Thus, pAVE2 is suitable for expression of cDNAs in cultured cells, where introns have little effect on gene expression. To test our new vectors, we inserted the structural region of the chromosomal *hGH* gene into pAVE1, and its cDNA into pAVE2. By independently transfecting the resulting recombinant plasmids into COS-7 cells, we have achieved high levels of *hGH* transient expression with both vectors.

INTRODUCTION

Bacterial plasmids modified by recombinant DNA techniques to facilitate expression of cloned genes or of their cDNAs in cultured cells are valuable tools for studies of eukaryotic gene structure and expression.

Earlier observations suggested that splicing was obligatory for mRNA accumulation in the cytoplasm (Hamer and Leder, 1979). Thus, the first generation of cDNA expression vectors usually included a heterologous intron in addition to the promoter and polyadenylation sequences (Mulligan and Berg, 1980). However, recent studies have shown that, in general, introns have little effect on the expression of genes transiently introduced into cultured cells (Brinster et al., 1988).

We searched for transcriptional control elements that could drive the expression in cultured cells of structural regions derived from cloned mammalian genes, more efficiently than the pair of expression vectors recently contributed by our laboratory (Cab-Barrera and Barrera-Saldaña, 1988). In a recent report, the hCMV enhancer-promoter unit was described as being one of the most powerful (Foecking and Hofstetter, 1986) and versatile units studied so far (Boshart et al., 1985). In the present work, we show the construction of a pair of new vectors

Correspondence to: Dr. H.A. Barrera-Saldaña, Unidad de Laboratorios de Ingeniería y Expresión Genéticas, Departamento de Bioquímica, Facultad de Medicina de la Universidad Autonoma de Nuevo Leon, Apdo. Postal 1563, Monterrey, N.L. (México 64000) Tel. (528)348-3251, ext 157; Fax (528)348-7229.

Abbreviations: bp, base pair(s); CAT, Cm acetyltransferase; *cat*, gene encoding CAT; Cm, chloramphenicol; hCMV, human cytomegalovirus; hGH, human growth hormone; *hGH*, gene encoding hGH; hPL, human placental lactogen; kb, kilobase(s); MCS, multiple cloning site; nt, nucleotide(s); *ori*, origin of DNA replication; RIA, radioimmunoassay; SV40, simian virus 40.

carrying this transcriptional control region. Their value for studies of gene expression is demonstrated here using them to transiently express the *hGH* gene sequences.

EXPERIMENTAL AND DISCUSSION

(a) Expression vectors

We compared the synthesis of CAT in COS-7 cells transfected separately with pSV2cat and pCMVcat (kindly provided by Dr. H. Hofstetter) using the DNA-Ca · phosphate co-precipitation technique (Graham and Van der Eb, 1973). The transfected cells were harvested for the CAT activity assay, 48 h after adding the precipitate (Gorman et al., 1982). As previously reported (Foecking and Hofstetter, 1986), we detected a much higher signal intensity from the acetylated forms of Cm produced by the extract of pCMVcat-transfected cells, as compared to that generated by the extract of those cells transfected with pSV2cat (data not shown). To exploit the advantages of the promoter strength and little tissue or species specificity (Boshart et al., 1985) provided by the hCMV enhancer-promoter unit, we decided to derive more versatile cloning and expression vectors from pCMVcat. The strategy for construction and the structure of the new expression vectors, pAVE1 and pAVE2, are illustrated in Fig. 1.

Vector pAVE1 was derived from pCMVcat by replacing the *Eco*RI-*Hin*dIII *cat* fragment with an analogous fragment of pUC19 MCS (Yanisch-Perron et al., 1985). This new plasmid harbors eight unique restriction sites (*Hin*dIII, *Sph*I, *Bam*HI, *Sma*I, *Xma*I, *Ava*I, *Kpn*I and *Eco*RI) immediately downstream from the hCMV enhancer-promoter unit. This MCS confers to pAVE1 great versatility for inserting and placing promoter-less chromosomal genes under the transcriptional control of the powerful hCMV enhancer-promoter unit.

Since introns have little effect on the expression of genes transfected into culture cells (Brinster et al., 1988), we decided to construct a second cloning vector for the transient expression of cDNA sequences. As illustrated also in Fig. 1, pAVE2 was derived from pAVE1 by incorporating the 3′-end flanking region from the *hGH* gene into the latter. This region includes sequences for primary transcript 3′-end processing and polyadenylation. Between the *hCMV* and *hGH* gene sequences, pAVE2 has six unique cloning sites: *Hin*dIII, *Sph*I, *Bam*HI, *Sma*I, *Xma*I and *Ava*I.

(b) Production of hGH by pAVE1 carrying the *hGH* gene

To test the ability of pAVE1 to express mammalian genes, we inserted the promoter-less chromosomal *hGH* gene, from a *Bam*HI site (at nt + 2) to an *Eco*RI site (located about 600 nt downstream from the polyadenylation signal), between the same sites in pAVE1 (Fig. 1).

pAVE1hGH was transfected into COS-7 cells, and 48 later total RNA was isolated by the thiocyanate–phenol–chloroform technique (Chomczynski and Sacchi, 1987). Northern-blot analysis (McMaster and Carmichael, 1977; Thomas, 1980) of this RNA was carried out using a random primer-labeled (Feinberg and Vogelstein, 1983) *hPL* cDNA as a probe (Barrera-Saldaña et al., 1982). The probe displays over 90% sequence similarity to *hGH* cDNA. The autoradiograph showed an RNA band hybridizing to the *hPL* cDNA probe (data not shown) with the expected size for the *hGH* mRNA.

To verify the correct expression of the transfected novel hybrid gene, the production of secreted hGH by the transfected cells was determined using a commercially available (Diagnostic Products Co., Los Angeles, CA) hGH RIA kit. This test was performed directly on the tissue culture medium to determine the extracellular concentration of hGH. Values of secreted hGH averaged 1.2 μg/25-cm^2 flasks of transfected cells. Although using different cell lines, when compared with results reported in the literature, our values of hGH production are very similar to those obtained by transiently transfected L cells (Selden et al., 1986). Forty-fold lower values of hGH production have been reported for Vero cells transiently transfected with a SV40-based vector carrying the *hGH* gene (Lupker et al., 1983).

(c) Expression of *hGH* cDNA in culture cells using the pAVE2 vector

The pAVE2 vector is designed to be suitable for expressing cloned cDNAs because it carries nt sequences necessary for 3′-end processing and polyadenylation of primary transcripts. The ideal candidate to demonstrate the expression properties of pAVE2 was the *hGH* cDNA, since we had already expressed its corresponding gene using pAVE1 and thus comparisons could be made.

For the above purpose, and because we did not have available the *hGH* cDNA, we designed a simple and rapid method which allowed us to almost selectively clone the cDNAs for both the 22-kDa and 20-kDa forms of hGH, simultaneously. Details of this method will be described elsewhere (D.E. R.-L. and H.A. B.-S., in preparation). We then proceeded to introduce the *hGH* cDNA fragment corresponding to the 22-kDa form of hGH (hGH-22 K), into pAVE2. Since the cDNA copy we cloned is not full-length, we had to assemble an intron-less *hPL-hGH* hybrid gene. This gene was constructed using the following DNA fragments in a 5′ → 3′ direction: (*i*) the *hPL-3* gene (from the capping site to the *Pst*I site just before the end of the first exon); (*ii*) the *hPL-3* cDNA (from the *Pst*I site up to an *Aat*II site located at the beginning of the second exon); (*iii*) the *hGH* cDNA (from the *Aat*II site to the *Sma*I site

located 4 nt downstream from the stop codon); and (*iv*) the *hGH* gene from the *Sma*I site to the 3′ end of the gene (already present in the vector). In spite of this tailoring of gene pieces, the mature hGH protein derived from this hybrid gene is coded entirely by the *hGH* cDNA. The resulting hybrid was named pAVE2hGH22k.

After transfecting in parallel COS-7 cells with the pAVE2 derivative, as well as with the positive (pAVE1hGH) and negative (pAVE1) controls, we obtained total cellular RNAs and culture media and used them in Northern-blot

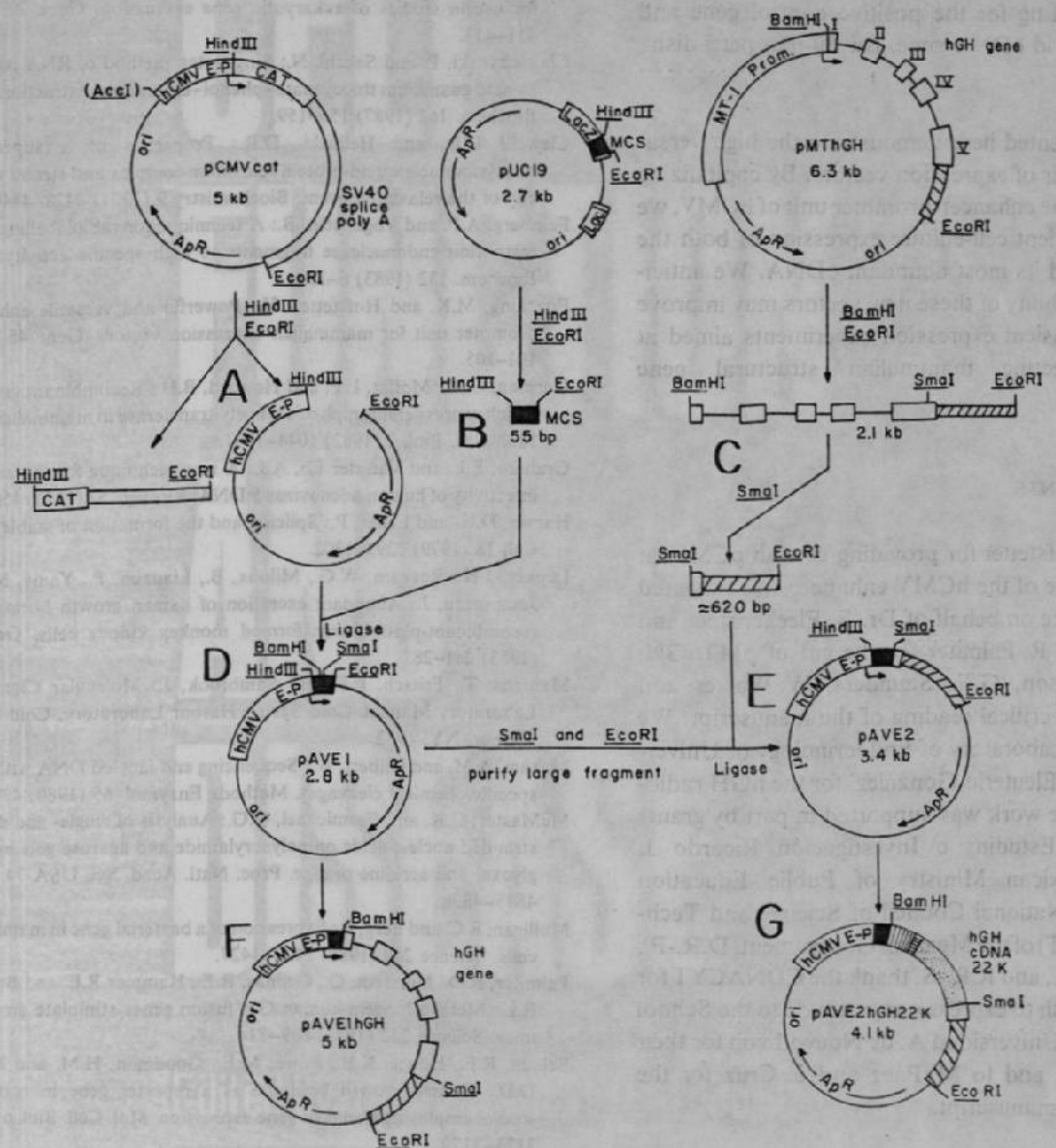

Fig. 1. Construction of the pAVE vectors and pAVEhGH derivatives. The plasmid pCMVcat (Foecking and Hofstetter, 1986), pUC19 (Yanisch-Perron et al., 1985) and pMThGH111 (Palmiter et al., 1983) were used for the construction of pAVE vectors and pAVEhGH derivatives. Restriction and modification enzymes were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, MD), New England Biolabs Inc. (Beverly, MA) and International Biotechnology Inc. (New Haven, CT), and used as suggested by the supplier. A series of DNA fragment isolations (**A**, **B** and **C**) were performed using standard enzymatic reactions (Maniatis et al., 1982) and protocols for DNA recovery (Maxam and Gilbert, 1980; Weislander, 1979; Vogelstein and Gillespie, 1979). The isolated fragments were then assembled in different combinations (**D** and **E**) in reactions catalyzed by T4 DNA ligase. The pAVE1hGH (**F**) was constructed from pAVE1 and the *hGH* promoter-less gene fragment (**C**). Finally, pAVE2hGH22K (**G**) was assembled from the different DNA pieces as explained in section **c**. Vertically striped box, hPL-3 gene 5′ end; horizontally striped box, hPL-3 cDNA; stippled box, *hGH* 22-kDa hGH; open and hatched boxes, *hGH* gene 3′ end. Sizes are not drawn to scale. Ligated materials were ethanol-precipitated, resuspended in 10 mM Tris · HCl pH 7.5/0.1 mM EDTA and used to transform *E. coli* strain RRI using standard protocols (Maniatis et al., 1982). Rapid preparation of plasmid DNA from 3 ml overnight cultures was done by the alkaline method (Birnboim and Doly, 1979) with modifications consisting of phenol (saturated according to Maniatis et al., 1982) chloroform–isoamyl–alcohol (25 : 24 : 1) extraction of the clear lysate, replacing the Na · acetate in the high salt solution for K · acetate and finally reprecipitation with ammonium acetate and ethanol. Large-scale preparation of plasmid DNA for transfection studies was carried out by the clear-lysate method (Clewell and Helinski, 1971) followed by centrifugation through a CsCl-ethidium bromide gradient.

analysis and hGH RIA, respectively. Both the transiently expressed natural (from pAVE1hGH) or hybrid (from pAVE2hGH22K) *hGH* mRNAs were easily and specifically detected by hybridization with the ^{32}P-labeled *hPL* cDNA probe (data not shown). The RIA values of secreted *hGH* averaged 691 ng for the positive control gene and 921 ng for the hybrid cDNA gene, per 60-mm petri dish.

(d) Conclusions

The results presented here demonstrate the high versatility of our new pair of expression vectors. By capitalizing on the strength of the enhancer-promoter unit of hCMV, we have achieved efficient cell-culture expression of both the *hGH* split gene and its most abundant cDNA. We anticipate that the availability of these new vectors may improve the efficacy of transient expression experiments aimed at functionally dissecting mammalian structural gene sequences.

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CELL FUNCTION AND DISEASE

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THE HUMAN PLACENTAL LACTOGEN AND GROWTH HORMONE MULTI-GENE FAMILY

Hugo A. Barrera-Saldaña[1,2], Ramiro Ramírez-Solís[1], William H. Walker[3], Susan L. Fitzpatrick[3], Diana Reséndez-Pérez[1] and Grady F. Saunders[3]

[1]Unidad de Laboratorios de Ingeniería y Expresión Genéticas (ULIEG) del Departamento de Bioquímica. Facultad de Medicina de la U.A.N.L., Monterrey, N.L. México. And [2]The International Center for Molecular Medicine, Monterrey, N. L. México

[3]Department of Biochemistry and Molecular Biology M.D. Anderson Hospital and Tumor Institute. The University of Texas System Cancer Center, Houston, Texas, 77030 USA

INTRODUCTION

The central problem of molecular biology is the understanding of how the genetic information coded in the nucleic acid is expressed and, what are the mechanisms that regulate such expression. Much progess has been made in understanding gene regulation in prokaryotic systems, however, in eukaryotic organisms, the advances have been much slower and much more recent. This is closely related in part to the degree of evolutionary complexity of the eukaryotic cell.

The regulation of genetic expression is an essential characteristic of living cells. Of the total amount of genetic informacion possessed in all cells, only a small fraction is differentially transcribed within a certain time and space.

Studies on the expression of procaryotic genes showed that besides the region of DNA to be transcribed into RNA, sequences located both to the 5' side (operators, promoters, etc.) and 3' side (terminators) of this structural gene, are involved in regulating gene expression. In addition, according to the operon theory (1), for the regulation of the expression of bacterial genes, various elements are required such as: DNA-dependent RNA polymerase (the enzyme responsible for transcribing the gene); several types of protein factors associated with the enzyme (σ rho, etc.) as well as metabolites (inducers, repressors, etc.) which interact with DNA to regulate bacterial gene expression (1).

Eukaryotic cells have approximately 10^3 times more DNA than a bacterium, multiple forms of DNA-dependent RNA polymerases (at least three types, each transcribing a different subset of genes) and compartmentalization of the processes of transcription (in the nucleus) and translation (in the cytoplasm). In addition to these differences, the discovery

in eucaryotes of split genes (2), and RNA processing, made it clear that regulation of eukaryotic gene expression is complex and can be exerted at a variety of different levels (3). The picture becomes even more complicated considering the organization of the eukaryotic genome into chromatin (4). In this regard, genes being expressed are said to be in an "active" chromatin configuration, although what determines this configuration is unclear.

THE PLACENTA AS AN EXPERIMENTAL MODEL

In evolutionary terms the placenta is recently acquired and a very efficient organ that functions to aid in the survival of the offspring and thus in the perpetuation of the species. Development of placental mammals allowed the mother to carry the unborn young with her while searching for food, thus protecting the fetus from predators. It also increased the area that could be covered during the searching process and facilitated migration. Thus, the placenta played a crucial role in the success of mammals colonizing the earth (5).

The placenta (6) is a remarkable organ in that is created from the same fertilized ovum that gives rise to the fetus but functions independently. The placenta exhibits unique characteristics that make it an excellent model for biological research. These properties include: a) its rapid growth and invasion of the maternal uterine tissue, b) the sudden stop of this invasion (by an unknown mechanism), c) the immunological processes that protect the placenta and the fetus from rejection, d) its hormonal regulation of pregnancy, etc.

In addition, the placenta is the organ with the highest rate of protein synthesis (7); and, since it develops and matures in less than 40 weeks, it constitutes an excellent system to study changes in gene expression during development and cell differentiation.

The placenta synthesizes a large variety of hormones (8). Probably the best characterized are chorionic gonadotropin (hCG) and placental lactogen (hPL, also know as chorionic somatomammotropin; HCS). While first trimester placental tissue is highly active in the synthesis of hCG with only low levels of hPL, in term placenta the situation is reversed (7). High levels of hPL (up to one gram per day) but low levels of hCG are produced by placenta at term.

In 1962, Josimovich and MacLaren (9) defined and characterized human placental lactogen as a polypeptide hormone present in extracts of human term placental and retroplacental blood that exhibited both potent lactogenic activity and an immunochemical reaction of partial identity with human growth hormone. hPL maternal blood levels are used to reflect the functional integrity of the placenta during pregnancy (10). hPL influences mammogenesis and lactogenesis as well as many aspects of the maternal intermediary metabolism directly related to the supply of nutrients for the metabolism of the fetus (11). However, the primary action of this hormone has not been defined.

The hPL molecule is a single-chain polypeptide of 191 aminoacids, produced in the syncytiotrophoblast layer (12). It contains two intramolecular disulfide bonds and no carbohydrate or lipid.

The hPL production is coupled to the development of the placenta, reaching its maximum towards the end of pregnancy. The great quantities in which this hormone is produced, makes this hormone ideal for research

and biochemical manipulations. For these reasons, we choose the placenta as the ideal organ to carry out the studies described in this paper.

RECOMBINANT DNA: A NEW AND POWERFUL TECHNOLOGY TO STUDY GENE STRUCTURE AND EXPRESSION

The birth of recombinant DNA technology in the early 1970's, marked the beginning of a new era in Molecular Biology. Recombinant DNA simply means the recombination in the test tube of different DNA molecules. This technology has provided us with very powerful tools and methods for the isolation, characterization and manipulation of gene sequences.

With the aid of this technology, it has been possible to begin the analysis of highly complex genomes of eukaryotic cells at the molecular level. Studies are being carried out to analyze the molecular structure and organization of genes in order to understand their function, regulation and origin.

The essential elements that constitute the group of recombinant DNA thechniques include:

I. Enzymes to modify DNA and RNA

Such as restriction enzymes, ligase, phosphatase, reverse transcriptase, DNA polymerase, polynucleotide kinase, etc. These proteins are employed to carry out the manipulation process of the DNA to be cloned. These genetic manipulations consist of specific cleavages along the DNA molecule or, modifications, covalent unions, radioactive labeling, etc.

II. Molecular Hybridizations

They can be performed using a liquid or solid support. This technique (13) consists of the detection, through the use of radioactive probes containing complementary sequences, of desired molecular species that are present in complex mixtures of DNA or RNA. These radioactive probes when denatured and later renatured in the presence of the mixture, form molecular hybrids with the desired single DNA chain or RNA. This coupling is stable due to the establishment of hydrogen bonds between the complementary nucleoside bases of the hybrid molecule.

III. Molecular vehicles

They include: plasmids (14), cosmids (15), lambda bacteriophage derivatives (16) and M13 bacteriophage derivatives (17). These are used to clone foreign DNA fragments (such as: human genes) and permit its propagation in bacteria; thus exploiting the following three qualities:

a. DNA fragments can autonomously replicate in host cells as they are inserted into vectors containing replication origins.

b. They can be separated from the bacterial nucleic acids and easily purified.

c. They contain DNA regions that are not essential for its propagation in bacteria. Foreign DNA inserted in these regions is replicated and propagated as if they were a normal component of the vector.

IV. Determination of the sequence and synthesis of DNA

This can be carried out either by enzymatic (18) or chemical (19) techniques. The sequencing methods for DNA (or including RNA) generate a great quantity of valuable information concerning the primary structure, organization, regulation, and evolution of the genes and proteins which they code. Thanks to these techniques, it is now much easier to determine the amino acid sequence of a protein through the sequence of its cloned messenger RNA (transformed to DNA through reverse transcription). On the other hand, it is possible to sequence a part of a protein whose gene we wish to isolate and characterize. With the information of the amino acid sequence and genetic code, it is possible to synthesize an oligonucleotide capable of serving as a probe to carry out molecular hybridizations. In this manner, the desired gene is isolated and characterized from a gene bank.

V. Gene library or bank

It is created (20) starting from either plasmids, bacteriophages, or cosmids; all containing either natural genes (gene bank) or DNA complementary (cDNA) to the messenger RNA population of a particular tissue or cell (cDNA bank). Basically, what is done with these banks is to take advantage of the classical bacterial models that have been so useful to elucidate the molecular basis of the regulation and expression in prokaryotics, to study the molecular genetics of higher organisms.

Briefly, to clone and isolate a gene, the following steps are carried out:

1. The genome under study is isolated
2. It is then fragmented by the use of restriction enzymes
3. The resulting DNA fragments are introduced in molecular vehicles, thus constructing gene banks.
4. A radioactive probe containing a complementary sequence to the gene portion that we propose to isolate, is used to identify the clone that contains the gene under study.
5. Sufficient quantities of the desired gene is purified.
6. The desired gene is characterized.

After carrying out these steps, the gene is used to perform the pertinent studies which help us understand the evolutionary history and gene functions in the living organism.

From the numerous studies carried out concerning the molecular structure and organization of the genes in eukaryotics, the term, split gene, has emerged (2). The majority of the genes in higher organisms are discontinuous. This means that the DNA which codes for the protein is interrupted by non-coding regions known as introns. These introns form part, as well as the code regions (or exons), of the primary transcript of the gene; but, are eventually eliminated to produce the mature mRNA which later is translated into a specific protein. In fig. 1, the molecular anatomy of the split gene model is outlined. Besides the introns and exons, some DNA regions or sequences are described. These DNA regions are also important to achieve a precise and efficient expression of these type of genes.

EXPERIMENTAL APPROACHES: THEIR STRATEGY AND INTERRELATIONSHIP

The experiments described in this section, were possible due to the development of Recombinant DNA techniques. Starting from the idea of exploiting the human placenta as the experimental model to study the mechanisms which regulate the specific genetic expression of the tissue, and using recombinant DNA techniques; our effort was focused towards the biogenesis of the most abundant protein in this organ, the Placental Lactogen hormone (hPL).

The particular objectives of our experiments were to identify the components and to elucidate the different steps involved in the genetic flow of information responsible for the synthesis and regulation of this polypeptide hormone.

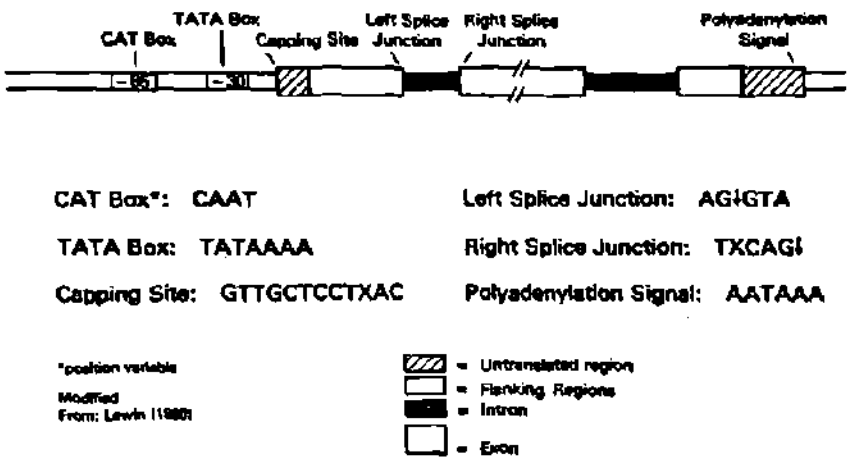


Fig. 1. Canonical protein-encoding mammalian gene. The figure shows the structural and regulatory sequences that characterize this type of genes.

In 1978, we started to investigate the genetic expression of hPL by trying to isolate and characterize the DNA dependent RNA polymerase type II of human placenta. This enzyme is responsible in carrying out the transcription of the genes that code for proteins. Because of this, it plays a key role in the functioning of the cell. However, this approach was somewhat premature since we first had to establish how to isolate and then biochemically and structurally characterize placental nuclei, which were to be our source of the enzyme. Once we accomplished this requisite (21) the isolation of the enzyme turned out to be a difficult project. Besides the inherent difficulties related with the technical procedures to be used, problems related with endogenous proteases and a lack of knowledge of the quaternary structure, were added on to our already existing problems. In spite of this, we were able to partially purify the enzyme and at least four of its subunits were detected (22). This was achieved through the use of electrophoresis in polyacrylamide gels with sodium dodecil sulphate (SDS).

With the coming of the DNA recombinant technology, the manner to attack the problem acquired a new focus. The molecular cloning of genes of higher organisms and the use of genetic engineering caused a revolution in the laboratories dedicated to the study of the gene expression and regulation. With a new plan in mind, adding these new techniques to the existing ones in our laboratory, we began to identify the elements that were involved in the different regulation levels of the biosynthesis of hPL (23). Briefly, we will mention our experimental strategy which can be used as a useful guide to present similar studies.

1) The in vitro synthesis of hPL and of its precursor form (pre-hPL) was studied (24).

2) The mRNA and pre-mRNA's for hPL were characterized (24).

3) The construction of a cDNA bank (DNAs which were copied from mRNA using the reverse transcriptase enzyme) was performed using terminal human placenta mRNAs (24).

4) The cDNA derived from the hPL mRNA was isolated (24).

5) This cloned cDNA was characterized, and sequenced (25).

6) The chromosomal localization of the hPL and hGH genes was carried out by in situ hybridization (26).

7) The number of genes for these two hormones was estimated (26).

8) The nuclear genes (27) for these hormones were isolated from a library of the human genome.

9) The cloned cDNA was hybridized against cloned hPL genes to form heteroduplexes (i.e. double stranded DNA molecules formed between one strand of cDNA and the complementary strand of the gene). The examination of these hybrid DNA molecules with the electron microscope, revealed the presence of four small introns in the hPL genes (24).

10) Two genes for hPL, two for hGH and one hPL-like gene were identified (23).

11) The expression of the genes for hPL were analyzed (25).

12) The hPL - like gene was sequenced showing similarities with the genes for hPL.

13) Elements involved in regulation of hPL transcription were identified.

The experimental projects and their results are described as follows:

MOLECULAR STRUCTURE

Molecular cloning of the mRNA for hPL

Using the same experimental model (the placenta) but with more powerful methods at hand, we proposed new and more ambitious questions:

Why is it that the placenta at term contains four to five times more translatable mRNA for hPL than first trimester placenta?. What were the

possible mechanisms involved in the specific regulation of the expression of the gene (or genes) for hPL?.

In an effort to respond to these questions, we decided to study the structure, abundance and origin of the mRNA for hPL.

Projects carried out by other investigators (28) indicated that the mRNA for hPL should contain approximately 900 nucleotides. In addition, a fragment of 550 base pairs corresponding to a portion of DNA complementary to the hPL mRNA (synthesized using reverse transcriptase and the DNA polymerase I of Escherichia coli) was already cloned in plasmid (29).

We isolated the total nucleic acids from term placenta. The high molecular weight RNAs were purified by the use of selective precipitation with 3M sodium acetate, pH 5.2. From these RNAs, the mRNA were selected through affinity chromatography in columns of oligo-dT-cellulose. This was achieved by exploiting the characteristic property of mRNA's possessing poly A "tails" in the 3' end.

The RNA messengers were translated in a cell-free system prepared from rabbit reticulocytes and mouse cell cultures. The synthesized proteins (which were radioactively labeled) were analyzed by the use of electrophoresis in polyacrylamide gels with SDS. By carrying out immunoprecipitation using anti-hPL serum two bands were observed: one that co-migrated with purified hPL and another more prominent, that most likely represented a pre-hPL (i.e. the immature form of hPL containing the signal peptide). The sum of these two bands represented approximately 15% of the total radioactively labeled protein.

When the RNA messengers were analyzed through electrophoresis in urea-acid-agarose gels, a prominent band of approximately 860 nucleotides was observed (24). A band of the same magnitude was observed when a recombinant plasmid, which contained the cDNA fragment of 550 nucleotides of hPL (30), was labeled with ^{32}P and hybridized against total RNAs fixed within filters. When the nuclear RNA was analyzed using this same method, four additional bands were observed of 990, 1200, 1460 and 1760 nucleotides. These most likely are the hPL mRNA precursors. The RNA messengers were also used to construct a cDNA bank.

Approximately 5% of the recombinant clones, which constitute the bank, hybridized with sequences of hPL DNA. This indicated that the RNA messenger for hPL is certainly abundant in terminal placental tissue. One of the clones which scored positive in the hybridization, contained a cDNA of approximately 815 base pairs. This clone was isolated and characterized by the use of restriction enzymes forming the map seen in fig. 2.

cDNA molecules were hybridized with molecules of the hPL gene to construct what is known as a heteroduplex. The electronic microscope analysis of the heteroduplex revealed the presence, in the hPL gene, of four small introns or intervenient sequences (fig. 3) which explains the presence and sizes of the four precursors for the hPL mRNA present in the nuclear RNA.

The importance of these results is that they established for the first time the following:

1. The complete map of the recognition sites of the restriction enzymes present in the complementary DNA of the mRNA for hPL.

2. The existence of precursors (pre-mRNA) of the mRNA for hPL.

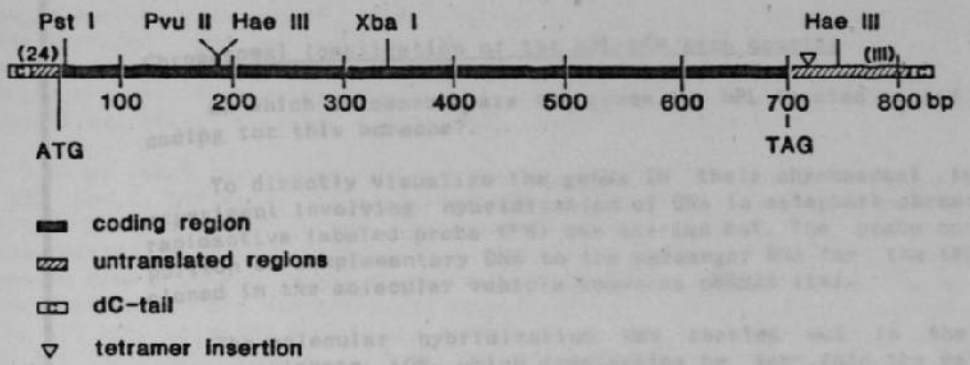


Fig. 2. Map and organization of the hPL cDNA insert of phPL815. It contains the 651 nucleotides (nuc.) coding for the 26 aminoacids of the signal peptide and 191 aminoacids of the mature hPL hormone. In addition, the insert also includes 24 nuc. of the 5' unstranslated region and 111 nuc. of the 3' untranslated region.

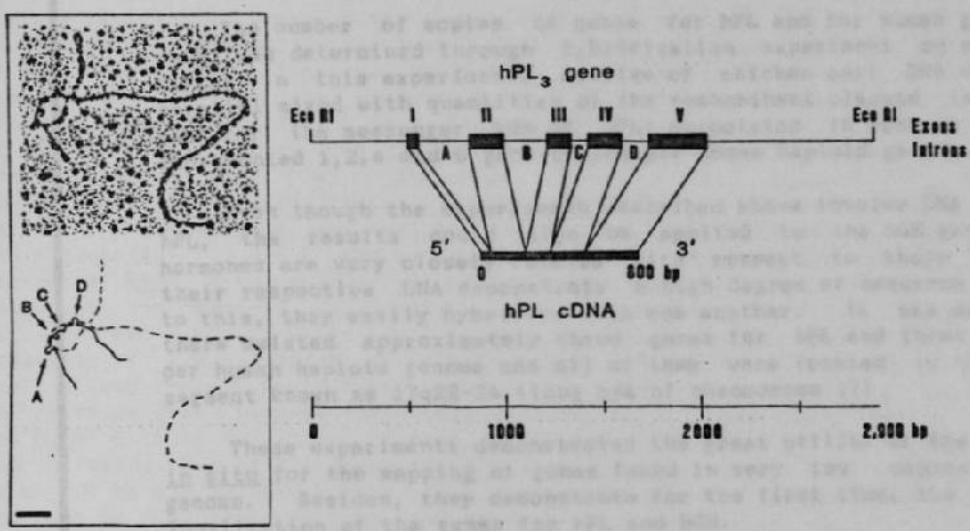
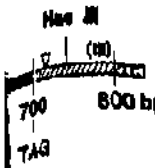


Fig. 3. Molecular structure of the human placental lactogen genes. The information obtained from the analysis of 16 heteroduplexes, as the one of the electron micrograph at the left, was used to determine the molecular structure of the human placental lactogen genes (represented by hPL_3). The heteroduplexes were formed by hibridization of plasmid DNA (phPL815, dashed line) containing the cDNA to hPL mRNA, with the DNA containing the hPL_3 gene (solid line). Four small intervening sequences are detected in the heteroduplex region at positions indicated by arrows labeled A-D. Magnification is indicated by bar length of 0.1 µm in lower left corner.



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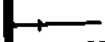

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3. The presence of four introns in the gene for hPL.

Chromosomal localization of the hPL-hGH gene complex

On which chromosomes are the genes for hPL located on and how many are coding for this hormone?.

To directly visualize the genes in their chromosomal location(s) an experiment involving hybridization of DNA in metaphase chromosomes with a radioactive labeled probe (^3H) was carried out. The probe consisted of a portion of complementary DNA to the messenger RNA for the (hPL) which was cloned in the molecular vehicle known as pBR322 (14).

The molecular hybridization was carried out in the presence of dextran sulphate 10%, which accelerates by ten- fold the velocity of the process of hybridization. Another important factor which decisively contributed to our success in the chromosomal localization of genes with very few copies per genome, was the presence of the DNA chains of the vector covalently linked to the insert. These single chain DNAs hanging from the site of hybridization where the insert is hybridized served as an anchor for multiple hybridizations between complementary chains of the denatured plasmid, resulting in an increased accumulation of specific radioactivity.

The number of copies of genes for hPL and for human growth hormone (hGH) was determined through hybridization experiment on nitrocellulose filter. In this experiment, samples of chicken cell DNA were tested in parallel mixed with quantities of the recombinant plasmid (containing the cDNA for the messenger RNA of hPL) calculated in such a way that they represented 1,2,4 and 6 gene copies per human haploid genome.

Even though the experiments described above involve DNA sequences for hPL, the results could also be applied to the hGH genes. These two hormones are very closely related with respect to their evolution and their respective DNA demonstrate a high degree of sequence similary; due to this, they easily hybridize with one another. It was discovered that there existed approximately three genes for hPL and three genes for hGH per human haploid genome and all of them were located in the chromosomic segment known as 17q22-24 (long arm of chromosome 17)

These experiments demonstrated the great utility of the hybridization in situ for the mapping of genes found in very few copies in the human genome. Besides, they demonstrate for the first time, the subchromosomal localization of the genes for hPL and hGH.

Isolation and characterization of the members of the multi-gene family

Having verified the number, the chromosome localization, the molecular anatomy of the hPL and hGH genes, and counting with the complete messenger RNA for hPL made from cDNA and finally cloned; we proceeded to isolate clones from the human gene bank (27). These clones contained complementary sequences to a portion of the cDNA for the mRNA of hPL cloned (30) into pBR322. This recombinant plasmid, proportioned by Dr. Peter Seeburg, is known as pBR322-HCS, (HCS stands for Human Somatomammotropin, name also given for hPL).

Kidd took DNA from the recombinant plasmid, labeled it with ^{32}P, denaturalized it, and immediately hybridized it against phage recombinant DNAs originating from the gene bank. From a total of 900,000 plaques of

phages analyzed, the seven that resulted positive, were grown and their DNAs purified. The DNA obtained from each recombinant phage, was characterized using restriction enzymes and hybridized against the hPL cDNA to locate regions containing hPL or hGH genes. Seven different genes were identified: hPL_1, hPL_2, hPL_3, hPL_4, hGH_1, hGH_2 and hGH_3.

In at least two phages, we were able to verify the connection between a hPL and a hGH gene. In addition, in another phage, we confirmed the linkage between two hPL genes. This indicated that probably all the genes were related with each other; thus suggesting, that all of these genes evolved from a common ancestor and that they originated by mechanisms of gene duplication and diversion. In more recent studies we established that hPL_2 and hPL_4 were the same gene, reducing to three the number of genes for hPL (hPL_1, hPL_4 and hPL_3). Also, the existance of the hGH_2 gene was not confirmed.

The Gene Structure

The molecular structures of the genes were obtained by four different methods. These were: 1) hybridizations on nitrocellulose filters (13) of the labeled cDNA (probe), against DNA from the fragmented genes obtained by the use of restriction enzymes. 2) comparing maps that contained various restriction enzyme cutting positions carried out for every gene, 3) confirming information obtained by determining the nucleotide sequence of the regions that flank the initiation and termination points of the genes, as well as, the borders between the exons and introns. 4) and finally, obtaining formation from the literature concerning the sequences of the cDNA and the gene for growth hormone, described by other groups of investigators (31,32). Four of the genes showed a very high degree of nucleotide sequence similarity with each other, as well as quite similar restriction enzyme maps. However, by: 1) the presence of repeated sequences adjacently to the 3' end of the gene, 2) the length of the fragments flanked by EcoRI sites and 3) the presence of characteristic restriction sites, it was possible to distinguish and identify each of these genes fig. 4). For example, while the two hGH genes are contained within Eco RI fragments of 2.6 kilobases (kb), both contain repetitive sequences near their 3' end region. Furthermore, both have unique sites for BglII. Even though these genes possess these similarities, one of them (hGH_1) has only one BamHI site in its fourth intron. As another example, we can mention the following. Two of the genes for hPL are contained within fragments Eco RI of 2.9 kb and possess unique sites for both XbaI and BamHI. However, they can be distinguished from each other by the presence of one (hPL_3) or two (hPL_4) PvuII sites. As a final example, the fifth characterized gene (hPL_1) is contained in a EcoRI fragment of 6.5 kb, which can be cut with XbaI, liberating a fragment of 3.5 kb containing the gene. In addition, this gene is characterized by the absence of XbaI and BamHI sites.

Molecular Anatomy of the Multi-gene Complex

The results obtained through the in situ hybridization experiments indicated that the genes were grouped within the region between the bands q22 and q24 of chromosome 17. Due to the quantity of DNA contained within this chromosomal region (several millions of base pairs), little could we deduce concerning the organization of these genes. Thus various questions arise: how close are these genes with respect to each other? What is the spatial relationship that the hGH genes have with respect to one another and with the hPL genes? Is each gene transcribed from the same DNA chain thus maintaining the same sense of transcript direction; or, do members exist that are transcribed from the opposite chain?

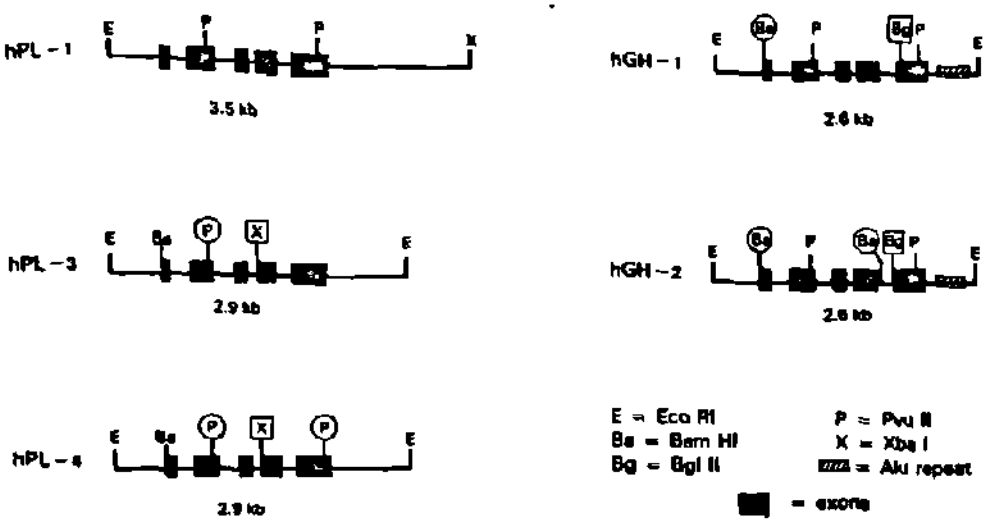

Fig. 4. The members of the hPL-hGH multi-gene family. Those enzymes useful to destinguish the different genes are in circles or boxes. hGH-1 is the same as hGH_N, while hGH-2 is also known as hGHv.

To solve these inquiries not only the data obtained from the isolated recombinant phages were analyzed; but, also we used information from other laboratories who were also working on the same theme. The data obtained from the molecular genetic analysis of patients who presented a congenital absence of hGH or hPL was also very useful to us. For example, a patient who presented with an absence of hPL (see Fig. 5), was shown to possess a deletion of approximately 35 kb with the loss of the genes hPL_1, hGH_1 and hPL_2. It was concluded that at least these three genes were quite close to each other. Having found recombinant phages containing two genes which were always one of the hPL type and the other of the hGH type, was particulary valuable in the establishment of the molecular anatomy of the genetic complex for hPL and hGH. This indicated that not only are the genes close to each other but, that they are also interwixed with respect to their spatial arrangement along the chromosome. In fig. 5, the map proposed for the genetic complex is illustrated. This map was later confirmed by isolating cosmid clones containing all the gene members of the hGH-hPL gene complex (33).

GENETIC EXPRESSION

hPL gene expression

What is the reason for the multiplicity of the hPL genes? What might be the function of these genes? Might it be that all are transcriptionally active in the term placenta or, are some of them pseudogenes? To answer these questions the following experiments were designed.

Fifteen recombinant plasmids were selected from a cDNA bank of term placenta RNA, that tested positive when they were hybridized to detect complementary sequences to hPL cDNA. Having knowledge of the map indicating the characteristic cleavage sites for the restriction enzymes of each hPL gene (see fig. 4); we proceeded to cut the DNAs of these 15 plasmids

with the restriction enzymes. This was performed with the objective of finding cDNAs corresponding to hPL transcripts of the hPL_1, hPL_2 and hPL_4 genes. By using this method, these genes could be distinguished by the absence or presence of certain sites of the diagnostic restriction enzymes for each gene. We were able to detect plasmids with insert characteristic of hPL_1 and hPL_2 but not however for the characteristics expected for a transcript of hPL_4. In this manner, we were able to establish for the first time, that hPL_1 and hPL_2 were transcriptionally active in term placenta; and that possibly, hPL_4 was a pseudogene or that it is expressed in other stages of the placental development. Both cDNAs were characterized and sequenced. Their representation in the term placenta, determined by three different experiments, was estimated to be in the proportion of two to three for the RNA messengers of hPL_2 and hPL_1 respectively.

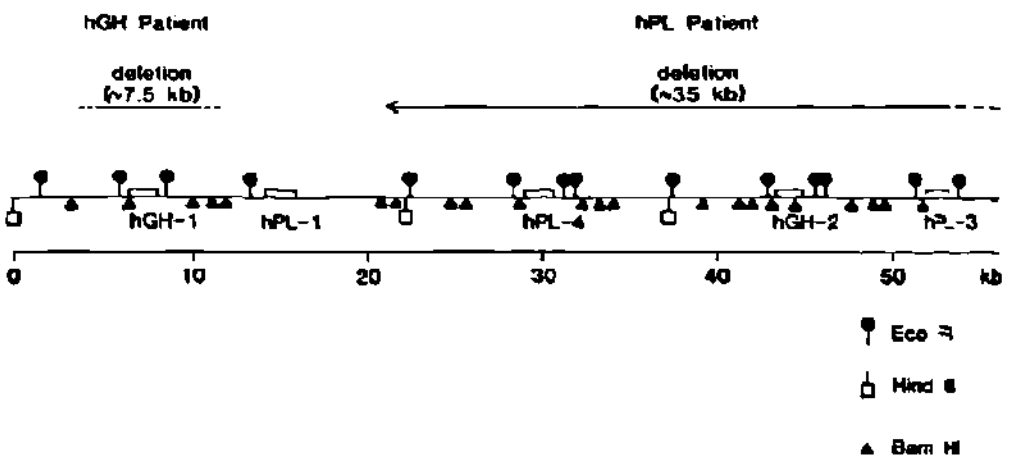

Fig. 5. Linkage map of the hPL-hGH multi-gene cluster. This map was constructed using information from restriction enzyme analysis of recombinant phages containing hPL and hGH genes, and the analysis of information from a molecular study of DNA from a patient with antenatal deficiency of hPL (see ref.46). The areas believed to be deleted in this patient and in a patient with familial isolated growth hormone deficiency are indicated above the map.

Finally, comparing the sequences of both cDNAs, 10 differences in the nucleotide positions were detected; although only one of them, caused a change in the amino acid sequence. This change is located in the signal peptide, which means that when this protein is processed before secretion, the mature protein coded by each of the two different genes is identical.

DNA sequences involved in regulation of the hPL gene.

Most cellular and viral genes have been shown to contain cis-acting sequences which regulate transcription of the gene. Some of these sequence include the TATA box and CAAT box located in the upstream promoter regions. Also hormone receptor sites, enhancers, and sequences which bind general or tissue specific factors can alter gene expression. These regulatory regions may be found upstream, downstream or within a gene.

We surveyed the entire hPL-hGH gene cluster for the presence of transcriptional enhancers (34). The gene cluster was digested with EcoRI and each restriction fragment was tested, using a transformation assay, for enhancer activity in hPL producing choriocarcinoma cells. The only enhancer detected was located 2 kb 3' to the hPL_3 gene (fig. 6).

A vector construct (fig. 7) containing the hPL enhancer linked to the reporter gene chloramphenicol acetyl transferase (CAT) under the control of the SV40 promoter was used in transfection studies to further characterize the hPL enhancer. These studies showed the enhancer was tissue specific as it was active only in cells which produce hPL. Transfection studies using choriocarcinoma cell lines have sublocalized the hPL enhancer to a 730 bp AccI-AvaI restriction fragment.

Deletion analysis of the enhancer suggests that multiple sequences throughout the 730 bp enhancer are necessary for enhancer activity. One region may be more important than others as over 60% of enhancer activity was found within the 5' most 210 base pares (bp). Protein binding studies suggest another 265 bp region (region II, fig. 6) may also be important. This region specifically binds protein found only in placental nuclear extracts. Regions I and III bind nuclear proteins common to both cells which produce hPL and cells which do not produce hPL.

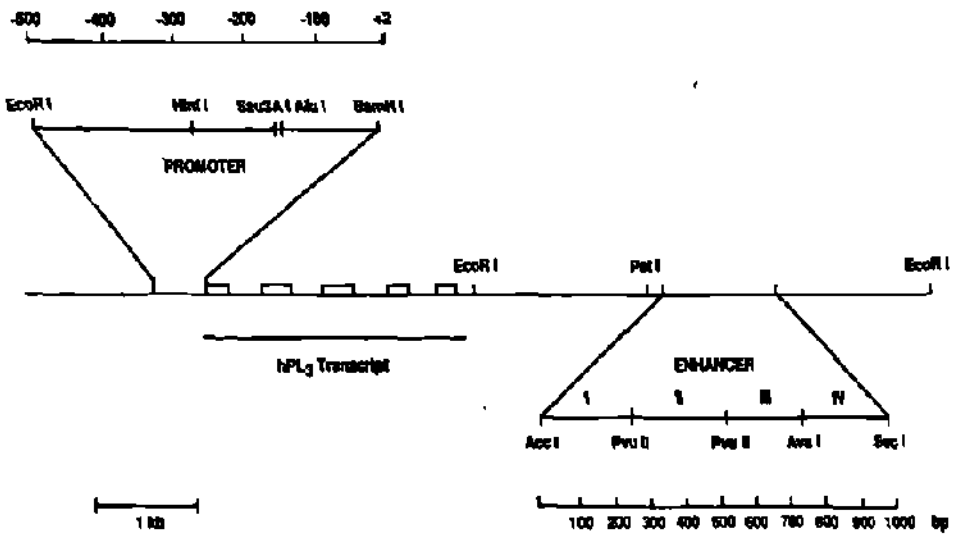

Fig. 6. hPL gene and regulatory sequences. Restriction enzyme digestion maps of both the promoter and enhancer regions are shown. Boxes correspond to hPL_3 exons. Enhancer restriction fragments tested for nuclear protein - DNA interactions are denoted as I, II, III and IV.

DNA sequences extending 500 bp upstream of the hPL and hGH genes are 95% homologous. When these 500 bp regions were inserted 5' to the CAT gene they were each shown to have low promoter activity. However, when the hPL enhancer was linked to either the hPL or hGH promoter, a marked difference in transcription activity was noted. Transcription activity of the hPL-promoter hPL-enhancer pair was 10-fold higher compared to that of the promoter alone, and 5 fold higher than the hGH-promoter hPL-enhancer

combination. This suggests that the hPL enhancer may act preferentially on the hPL promoter.

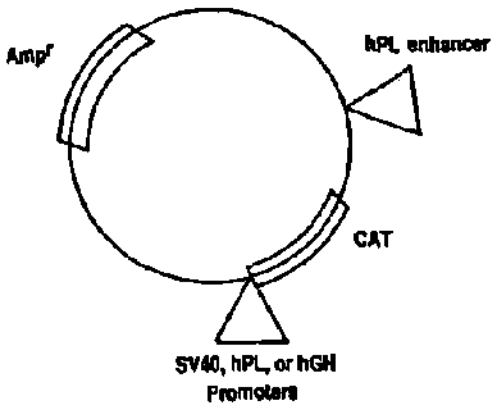

Fig. 7. CAT vector for hPL promoter and enhancer studies. Plasmid vector containing the promoter less bacterial gene for chloramphenicol acetyl transferase (CAT). Promoter sequences were inserted 5' to CAT and enhancer sequences added 3' to CAT.

The hPL promoter was analyzed to determine sequences that are important for regulating the transcription of hPL. Deletion mutants were created by digesting the 500 bp hPL promoter with various restriction enzymes. The resulting DNA fragments all contained the same 3' end but varied in length of 5' sequence (fig. 8). The deletion fragments were ligated 5' to the CAT gene in a vector that also contained the hPL enhancer to increase activity (see fig. 7). The plasmids were transfected into placental choriocarcinoma cells and the level of CAT activity assayed to determine the transcriptional strength of each promoter mutant. Maximal activity was seen when the promoter contained 390 bp of DNA and decreasing the size of the promoter to 152 bp did not lower this high activity. Initial experiments showed lower activity when the promoter fragments contained 2300 bp or 500 bp of upstream DNA sequence. A pronounced decrease (7-10 fold) in CAT activity was seen when the promoter was reduced to 129 bp. This low activity was also seen when the sequences between -152 bp and -129 bp were removed from the 390 bp fragment (390 SA). A new 142 bp clone was prepared and it had activity similar to the 152 bp clone indicating that an important region for transcription is contained between the DNA sequences -142 and -129 bp. This sequence may be important for binding trans-acting factors which stimulate the transcription of the hPL gene.

The hPL genes contain sequences both 5' and 3' to the genes that are important for its transcriptional control. Further study of these regions may elucidate their mechanism(s) of action in the regulation of hPL expression.

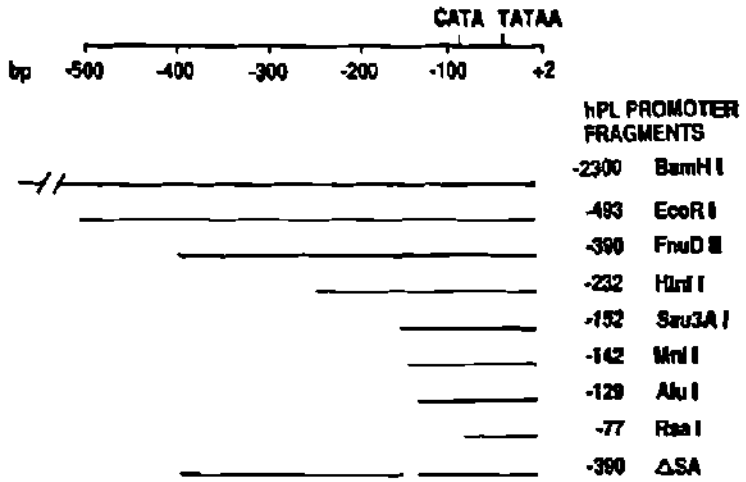

Fig. 8. Deletion mutants of the hPL promoter. Deletion mutants of the hPL promoter were created by digesting the DNA with various restriction enzymes. The fragments (solid line) all had identical 3' ends but decreasing amounts of 5' DNA.

Is hPL$_4$ a pseudogene?

What was the nature of the hPL$_4$ gene? What type of protein was coded in its nucleotide sequence? Was this gene, whose transcriptional products we could not find in term placenta, potentially functional? or did it contain mutations that rendered it as a pseudogene? To obtain an insight into these uncertainties, we decided to dissect the hPL$_4$ gene.

A 3.5 kb EcoRI - XbaI DNA fragment containing the hPL$_4$ gene was digested with various restriction enzymes. Aliquots of each of these digestions were ligated to M13 vectors that had already been cut with the appropriate restriction enzymes to generate compatible ends for cloning. Recombinants for each of the different fragments spanning the gene were identified and their single stranded DNAs were purified. The method of Sanger (18) was used to determine the sequence of the inserts in these M13 recombinants. The analysis of the hPL$_4$ sequence indicated that the characteristics of this gene are very similar to those of the other members of hPL-hGH gene complex. However, in spite of these similarities this gene possesses unique features. Both, the common and unique characteristics of the hPL$_4$ gene with regard to the other hPL genes are listed below.

1. It contains five exons of identical length to those in the other hPL and hGH genes.

2. It contains normal TATA (-30) and CAT (-84) boxes.

3. It contains a normal polyadenylation signal which is followed by a characteristic truncated Alu element located 100 nucleotides further downstream.

4. The protein that it encodes differs in 16 amino acid positions, as compared to the mature hPL protein coded by the hPL_4 and hPL_5.

5. The hPL_1 gene contains a G to A transition at the 5' splice consensus site of the second intron; thus, preventing pre-mRNA processing at this site and classifying hPL_1 as a pseudogene candidate.

When we searched for the presence of hPL_1 gene cDNA clones in a human term placenta cDNA library, we did not detect them (25). The search for transcripts of this gene by other group of investigators (35), who used hPL_1 gene specific oligonucleotides, was also unsuccessful. This evidence along with the presence of the point mutation at 5' splice site of the second intron of hPL_1, that in other genes (36) has been proven to be a cause of gene inactivity; strongly suggested that this gene was a pseudogene.

More direct experimental evidence was required to reach a definitive conclusion regarding hPL_1 gene expression. We decided to test in a transient expression experiment, if the splice site point mutation in hPL_1 rendered it unable to generate a mature mRNA. We rationalized that adequate controls for this experiment should be to introduce the mutation present in hPL_1 into a normal hPL gene and see if it now becomes defective in the production of mature mRNA, and to revert to wild type the mutation in hPL_1 to see if it now becomes active. The assumption behind these experiments was that the point mutation was the only cause of hPL-1 apparent gene inactivity. Next, we went on testing this assumption.

Comparisons of the nucleotide sequences around the region of the 5' splice donor site, contained in the active hPL-3 gene and the putative hPL-1 pseudogene, made it clear that we could easily exchange the mutated area between these two genes. We found PvuII and SacI sites located 30 base pairs upstream and 86 base pairs downstream of the mutation site, respectively. The sequences in this restriction fragment, of approximately 120 bp, differs among the two genes in only four nucleotide positions. In addition to the single point mutation in the 5' donor splice site, there were three other nucleotides sites within this region where these genes differ. The additional changes, however, are located inside the intron at 6,27 and 64 bp downstream from the exon-intron border, positions that could be taken as of little importance for the pre-mRNA processing.

We carried out the shuffling of this restriction fragment among hPL_1 and hPL_3 genes. To our advantage, the differences between these two genes inside the second intron sequences, were associated with an AluI site. Thus, the successful exchange of the PvuII-SacI fragment could be monitored by AluI digestions of the recombinant hybrid genes.

Furthermore, to leave no doubt of the identity of the recombined genes, we determined their nucleotide sequence in the area around the exchange site. In this manner we were sure we had our recombined and wild type genes to test our hypothesis.

We next constructed a new plasmid vector derived from pCMVCat (a plasmid containing cytomegalovirus enhancer-promoter control sequences fused to chloramphenicol acetyl transferase structural gene). This construction was shown previously to be a powerful expression system (37). Our new vector called pAVE-1 contains: pBR322 (14) sequences from the EcoRI site to the AccI site; the enhancer-promoter sequences of cytomegalovirus (37); and the polylinker region of pUC18 (38). We then

subcloned into pAVE-1 the structural regions of our test genes and were ready to assay their expression (fig. 9).

To determine the expression of our hybrid genes, we choose to introduce them into cultured cells (transient expression-assay) by the technique of calcium-phosphate-DNA coprecipitation (39). The efficiency of transfection was evaluated by using the CAT assay carried out on a fraction of the cultured cells cotransfected with both the test gene and pCMVCat.

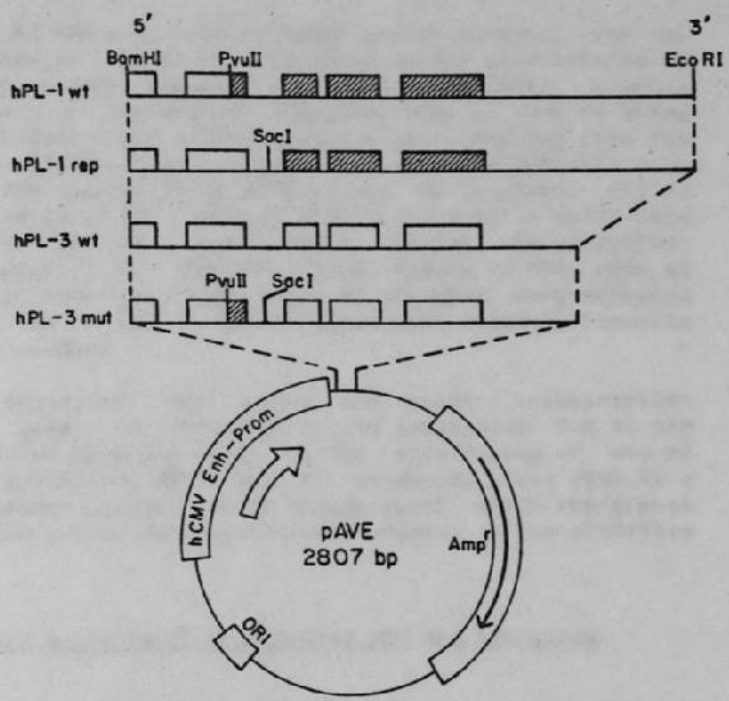

Fig. 9. Subcloning of hPL genes into the expression vector. The hPL$_1$ genes were directly cloned in pAVE-1 from their Bam HI site at beginning of the first exon to the Eco RI site at the 3' end of the gene. A different strategy was used for the hPL-1 genes since they lack the Bam HI site. To clone them, we replaced the 5' end of these genes with that of the hPL-3 gene, from the Bam HI site to the Pvu II in the second exon.

We isolated total RNA from the remaining transfected cells by the method of guanidinium isothyocianate-lithium chloride (40). Then we glyoxalated the RNA and separated it in an agarose gel. We transferred the RNA to a nitrocellulose membrane and hybridized it with an oligolabeled hPL cDNA probe. The preliminary results from this type of analysis indicated that the wild-type hPL-1 gene is not capable of producing a mature mRNA and that when its mutated splice donor site is substituted with a normal one this gene deffect is reversed. On the other hand, the wild-type hPL-3 gene produces good levels of hPL mRNA and which are

drastically decresed when this gene harbors the mutation present in hPL-1 gene.

Therefore, we concluded that hPL-1 is a pseudogene because, even when it has a high similarity to the hPL active genes, does not produce a mature mRNA.

hGH gene expression

Even though the 22 kilodaltons form of human growth hormone, the one which normally circulates in the blood, is coded by the gene located in the 5' region of the multi-gene complex (the hGH_n, or hGH_N, n being "normal"). The expression of the second hGH gene, hGH_2 or hGHv (v being "variant"), has been detected using an expression vector derived from the monkey simian virus 40 (SV40). When cells in culture are infected with this recombinant virus the transcription of gene can be observed; and as result, there is a production of a hormone that differs in 13 amino acid positions with respect to hGH_1 (41). More recently, by the use of synthetic oligonucleotides specific for the hGH_2 gene, levels of hGH_2 mRNA at least four orders of magnitude lower than those of hPL mRNA, were detected in term placenta (42). Up to now, it is not known what possible function may have this hGHv gene product.

The complications do not end here since the primary transcription product of the hGH_1 gene, is regulated in its processing. Due to the presence of two alternative splicing sites in the elimination of one of its intron (43); the additional mRNA that is produced, gives rise to a variant of the normal growth hormone (of 20 kilodaltons), which represents approximately 10% of the growth hormone activity present in the pituitary gland.

Expression and functional analysis of transfected hGH and hPL genes

hGH and hPL are the two well characterized products of the hGH and hPL multigene complex. They share 85% of their aminoacids. hGH is unique among the animal growth hormones in that it possesses prolactin-like activity. hPL also shows prolactin-like activity and in spite of its sequence similarity to hGH, it is virtually inactive as GH. The structural similarities and functional differences of these hormones offers a good opportunity to study the evolution of functional domains in proteins.

We are interested in studying the possible functions of the less characterized members of this multigene complex. While the major product of the hGH_n gene (the 22 kd form of hGH) and the identical mature protein encoded by both hPL_1 and hPL_3 genes are easily obtained from pituitary gland and placenta, respectively, this is not true for the putative protein products of the remaining gene members. The proteins encoded by the hGHv and the hPL_2 genes, and any other minor products generated through differential splicing from any of the gene members can only be studied by recombinant DNA approaches.

As a neccesary step to pursue our objetive we decided to stablish an efficient expression system based in the transfection of genes into cultured cells. Starting with the hGH_n gene, we have constructed new hybrid genes (Fig. 10) by joining the structural region of this gene with various types of transcriptional control elements. The novel joints plasmids harbor the following promoter and/or enhancer elements: the mouse metallothionein I promoter (pMThGH), the mouse metalothionein promoter together with the SV40 enhancer (pNUThGH), the promoter-enhancer of the

immediate early gene of human cytomegalovirus (pCMVhGH), as well as the natural promoter of the gene in conjunction with the SV40 enhancer (pSVgpthGH). As a result, we have achieved a good level of expression of this structural gene by transfection into COS-7 cells. Both Northern blotting and radioimmunoassay were performed to evaluate the strenght of these transcriptional control elements and hormone secretion, respectively. The best secretion of hGH was achieved using the pNUThGH plasmid. The potency of the remaining novel joints can be ordered, going from the strongest to the weakest as follows: enhancer-promoter of CMV, natural promoter in combination with the SV40 enhancer, and finally, we obtained the lowest secretion of hGH into the medium when using alone the promoter of MT-I.

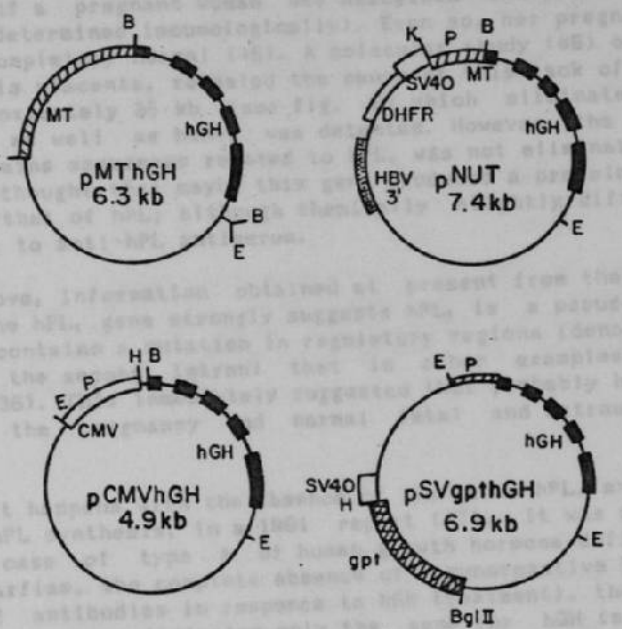

Fig. 10. Structure of hGH expression vectors. With the exception of pSVgpthGH, which contains the entire hGH gene (including its natural promoter), all the other vectors shown here were constructed by fusing the hGH gene structural region (BamHI site at the beginning of the first exon to EcoRI site at the 3' end) to various transcriptional control elements. In pMThGH expression of hGH promoter-less gene is directed by the mouse metalothionein promoter. In pNUT act both SV40 enhancer sequences and mouse metalothionein promoter. Finally, in pCMVhGH the transcriptional control element is the enhancer-promoter of the inmediate early gene of human cytomegalovirus.

Our results confirm those obtained by Pavlakis et al (41). Using SV40 vectors carrying the structural region of the hGH$_N$ and hGHv genes, they

showed that monkey kidney cells were able to process hGH prehormone and secret the mature hormone into the culture medium. The amount of hGH secreted in our transient expression experiments was comparable to that secreted by Vero cells lines permanently transfected with a plasmid carrying an SV40-hGH hybrid gene (44). Therefore, we are now ready to produce enough quantities of the other protein products encoded by the hPL-hGH gene family members, to start an analysis of their possible functions.

MOLECULAR BASIS OF THE DEFICIENCIES OF hGH AND hPL

In 1982, a case of a pregnant woman was described who presented a total absence of hPL (determined immunologically). Even so, her pregnancy, birth and child were completely normal (45). A molecular study (46) of the hPL-hGH complex in this placenta, revealed the cause of this lack of hPL. A DNA deletion of approximately 35 kb (see fig. 5) which eliminated the hPL_A and hPL_B genes, as well as hGHv, was detected. However, the fifth gene (hPL_F) which contains sequences related to hPL, was not eliminated in the deletion. It was thought that maybe this gene produced a protein with activities similar to that of hPL; although chemically slightly different since it did not react to anti-hPL antiserum.

As described above, information obtained at present from the molecular dissection of the hPL_A gene strongly suggests hPL_A is a pseudogene. This is because it contains a mutation in regulatory regions (donor site for the processing of the second intron) that in other examples would inactivate the gene (36). This immediately suggested that probably hPL may not be necessary for the pregnancy and normal fetal and extrauterine growth.

Contrary to what happens with the absence of the genes hPL_A and hPL_B responsible for the hPL synthesis, in a 1981 report (47), it was demonstrated that in a case of type A of human growth hormone deficiency (characterized by dwarfism, the complete absence of immunoreactive hGH and by the generation of antibodies in response to hGH treatment), the cause was a deletion of 7.5 Kb that eliminates only the gene for hGH (see fig. 5).

MOLECULAR EVOLUTION

The sequence analysis of the genes has given us valuable information to allow us to try to reconstruct the evolutionary process of the structure of the hGH-hPL complex. In 1971, Nial et al. (48) noticed the presence of similar aminoacid regions in the growth, placental lactogen and prolactin hormones. This observation indicated that these hormones were probably related to each other with respect to their evolution. This was also suggested by their physiological activities, since the three show overlapping activities in different degrees. These observations lead to the hypothesis the genes for these hormones constitute a family, from an evolutionary point of view, having originated from a common ancestor. This common ancestor, which is believed to be similar to prolactin (since it exists in all vertebrates while the other two hormones evolved later in higher vertebrates and placental mammals), underwent a gene duplication and subsequent divergence of the products. This implies that there existed at least two events of genetic duplication during the evolution of these hormones (fig. 11). The first established the branches of prolactin and of growth hormone. The second, most likely occurred in the growth hormone branch, giving rise to the placental lactogen hormones, the youngest member of this family.

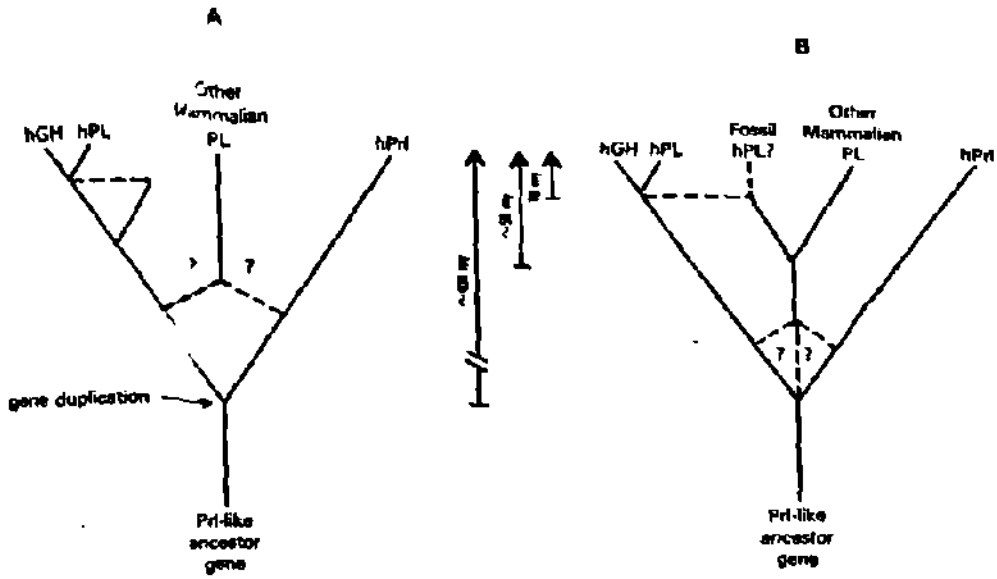

Fig. 11. Possible pathway for evolution of hPL in mammalians. Two alternative hypothesis are presented. In both of them a gene conversion mechanisms has rendered the aminoacid sequences in hPL and hGH very similar (85%).

Using taxonomic, amino acid, and nucleotide sequence comparisons, it has been estimated that the first genetic duplication occurred approximately 400 million years ago. This estimation goes back to the times when the evolutionary divergence of fish and tetrapods occurred; since the pituitary glands of amphibians, reptiles and birds contain different molecules similar to prolactin and growth hormone.

Estimations of the evolutionary origin of the hPL gene, using two different criteria, give conflicting results. The necessity of placental lactogen accompanied the origin of the principal orders of placental mammals, approximately 75 million years ago. On the other hand, the branching of the primates occurred about 60 million years ago, considered to be the approximate time when the hPL appeared. However, the analysis of the amino acid and nucleotide sequence suggest that the appearance of an hPL gene by duplication from its ancestor hGH gene occurred approximately 10 million years ago. To resolve this paradox, it has been postulated (49) that gene conversion must have occurred (a non-reciprocal, recombinant mechanism where a gene serves as a mold to correct mutations in another gene). The fact that the genes for these two hormones are contiguous on the same chromosome, could have facilitated this gene conversion; furthermore this event must have happened about 10 million years ago (see fig. 11).

Finally, the observations of the internal homologies of these three hormones, suggest that the common ancestral gene could have resulted from a repetitive duplication process of a gene that coded for a primordial peptide of approximately 20 amino acids (48).

PERSPECTIVES

The results presented here allow us to elucidate, at a molecular level, the route of the genetic flow of information for the Placental Lactogen hormone. Since its starting point in the nucleus of human placental cells and through the different levels of expression, this genetic information is a product of the structure, function, and evolution of this hormone.

Now we know the structure of the two active genes responsible for the production of the hPL. They are relatively small genes (the sequences present in mRNA are approximately 800 bp distributed in five exons interrupted by four small introns). These two genes are within a cluster that includes two genes for the growth hormone and a fifth gene that contains sequences that identify it as similar to the hPL genes. These genes are distributed in approximately 50,000 bp of DNA in the q22-24 region of the human chromosome 17.

In spite of the advances achieved, there is still much to be investigated concerning the expression and regulation of this genetic complex. We know nothing about the expression of the hPL genes during pregnancy nor in trophoblast pathologies. We are just begining to understand the mechanisms (in spite of the proximity and very high similarity of its genes) by which the cell manages to control the specific expression of the genes such that hPL is produced in the placenta, while hGH be produced in the pituitary gland. The causes of sudden increase of hPL synthesis during the second trimester, as well as the regulation of the synthesis of hPL are still unknown. We do not know if hPL is involved in the mechanisms of initiation of labor during birth, nor if it has any role in the immunological concealment that the placenta and fetus possess to avoid being rejected by the maternal immune system.

It is necessary to perform more definitive studies in relation to the expression of the hGHv gene, to better understand physiological role that its gene products might have.

It is clear that the studies described in this assay, allow a better understanding of the molecular basis that control biological processes so important such as cellular differentiation and development. A better understanding of these normal processes would help clarify the causes of genetic and other type of ailments, the aging process, and the origins of cancer.

Certainly, this is a good model to carry out studies related to genetic regulation and we hope to continue learning from it.

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UNIVERSIDAD AUTONOMA DE NUEVO LEON
FACULTAD DE MEDICINA
APDO. POSTAL MONTERREY, N. L. MEXICO

Unidad de Laboratorios
de Ingeniería y Expresión Genéticas
(U.L.I.E.G.)

july 19, 1991

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We hope our paper fullfills the criteria for acceptance. We would greatly appreciate reaching us by Phone, Fax, E-mail or by mail to our address in McAllen, Texas, because our postal service here in Mexico is slow.

Thank you very much for all your kind attentions and we hope to hear from you soon.

Sincerely yours,

Hugo A. Barrera Saldaña, Ph.D.
Phone: (528)333 7747
Fax: (528)348 7884
E-mail: HBARRERA@MTECV1.BITNET
Address: 2609 Quebec Street
McAllen, Texas 78501

Functional expression of human pituitary growth hormone complementary DNAs.

(Recombinant DNA, hGH DNAc; differential splicing; transient expression; eukaryotic expression vector; adipogenic activity)

Diego E. Rincón-Limas, Diana Reséndez-Pérez, Luis E. Alvidrez-Quihui, Federico Castro-MuñozLedo*, Walid Kuri-Harcuch* and Hugo A. Barrera-Saldaña.

U.L.I.E.G.-Departamento de Bioquímica, Facultad de Medicina, U.A.N.L. Monterrey, N.L., 64460 , México
*Departamento de Biología Celular, CINVESTAV-IPN México, D.F. 07000 México

Correspondence to: Dr. H.A. Barrera Saldaña.
U.L.I.E.G. Departamento de Bioquímica
Facultad de Medicina de la U.A.N.L.,
Apdo. Postal 1563, Monterrey, N.L.,
México 64000
Tel. (528)333 7747
Fax: (528)348 7884

Abbreviations: bp, base pair(s); hGH, human growth hormone; hPL, human placental lactogen; kb, kilobase pair(s); RIA, radioimmunoassay; met hGH^{bac}, recombinant hGH.

SUMMARY

We have isolated, cloned and assayed the functional expression of pituitary cDNAs for human growth hormone (hGH). A selective cDNA cloning strategy was used to preferentially obtain both hGH 22K and hGH 20K cDNAs. These were used to construct minigenes which were subcloned into the eukaryotic expression vector pAVE2 [Ramírez-Solís et al., Gene 87 (1990) 291-294]. Transfection assays in COS-7 cells of both minigenes proved they are similarly efficient in generating mature RNAs, and allowed the detection and visualization of the secreted hGH-22K and hGH-20K polypeptides. Finally, the hGHs transiently expressed and secreted into the culture medium by the transfected COS-7 cells were able to specifically promote differentiation of preadipocytes 3T3-F442A to adipose cells.

INTRODUCTION

The growth hormone normal (hGH-N) gene is the only member of the five gene human growth (hGH)-placental lactogen (hPL) hormone multigene family that is expressed in the pituitary gland [1]. In spite of its high (~85%) aminoacid sequence similarity with hPL; hGH has, as one of its distinctive functional features not shared by hPL, the capacity to induce differentiation of adipocytes [2].

Differential splicing of the hGH-N gene primary transcript generates two different mRNAs. The translation product of one of these mRNAs is the well characterized 22-KDa form of hGH (hGH-22K). The other mRNA contains a 45bp internal deletion and encodes the 20-kDa form of hGH (hGH-20K) [3-4]. The 20K hGH mRNA and its corresponding hormone are present at very low abundance in the pituitary gland.

In vitro mutagenesis [5] and molecular cloning experiments [4] have led to the isolation of the 20K hGH cDNA. Biological activities of the 20K-hGH have been found to be equipotent to 22K-hGH in various growth-promoting assays such as tibial tests, body weight gain tests and in somatomedin synthesis [6-8]. However there is controversy related with their effects on carbohydrate and lipid metabolism. Our understanding of the molecular biology, physiology and evolution of these alternative hGHs forms, will depend on the future supply of the

recombinant versions for comparative studies.

Here we report isolation, cloning and functional expression of both pituitary hGH cDNAs. We have integrated very simple cloning and production strategies, and evaluated the in vitro adipogenic activity of the cDNAs encoded hormones.

MATERIALS AND METHODS

Restriction and modification enzymes were purchased from Bethesda Research Laboratories Inc. (BRL, Gaithersburg, MD) and New England BioLabs Inc. (Beverly, MA), and used as suggested by the supplier. [$\alpha^{32}P$]-dCTP, ^{35}S-dATP and L-[^{35}S]-methionine were purchased from Amersham Intl. (Buckinghamshire, England). The enzymatic kit for cDNA synthesis was obtained from BRL and the sequencing kit (sequenase) was purchased from United States Biochemical (Cleveland, OH)

Total cytoplasmic RNA was isolated from pituitary gland by the thiocyanate-phenol-chloroform technique [9]. Quantity and quality of RNA preparations were determined spectrophotometrically and corroborated by agarose gel electrophoresis [10]. Poly(A)$^+$ RNA was subsequently selected by oligo(dT)-cellulose chromatography [11]. The poly(A)$^+$ RNA was converted to cDNA, digested with AatII and XmaI and cloned into the similary cut pUC19 plasmid. DNA samples obtained during the procedure were

electrophoresed in a 1.4% agarose gel, transferred to nylon membrane (Gene Screen Plus) and analyzed by Southern blotting [12] with an homologous ^{32}P-labeled [13] hPL cDNA probe [14].

DNA restriction fragments were purified from agarose or polyacrylamide gel slices. This was performed by electroelution or Geneclean (BIO101, La Jolla, CA) extraction [15]. Ligations, bacterial transformations and plasmid DNA isolations were carried out using standard protocols [16]. Recombinant plasmids carrying hGH cDNAs were characterized by restriction analysis and nucleotide sequencing [16].

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, (HyClone Laboratories, Inc., Logan, UT). They were maintained at 37°C with 5% CO_2. Plasmid DNA (7.5 µg/ 25 cm^2 culture flask) was transfected by the calcium phosphate method [17].

Northern blot analysis of total RNA obtained from transfected COS-7 cells and in vivo labeling of newly synthesized and secreted proteins were performed as previously outlined [18]. Quantification of hGH was achieved using a commercially available hGH radioimmunoassay (RIA) kit (Diagnostic Products Co., Los Angeles, CA). The test was performed as described [19]

directly on the tissue culture medium.

Samples of culture medium from COS-7 cells transfected with the different plasmids, were added to a final concentration of 30% (v/v) to confluent 3T3-F442A cell cultures supplemented with non-adipogenic medium [20]. After 7 days, cell cultures were fixed and stained with oil Red O for adipose conversion quantification. Control cultures were also maintained with non-adipogenic medium with or without 25 ng/ml met hGH ^{var}.

RESULTS AND DISCUSSION

Preferential cloning of pituitary hGH cDNAs.

The computer aided inspection of published hGH cDNA sequences [21] resulted in the identification of unique restriction enzyme sites flanking the hGH gene coding region. At the 5' end of both hGH cDNAs, we chose an AatII site centered at codon -20 of the signal peptide (26 aminoacid aminoterminal extension). At the opposite end, we found a convenient XmaI site located four nucleotides after the stop codon. The ligation of pituitary cDNA digested with AatII plus XmaI, to the similarly cut large DNA fragment of pUC19, resulted in the integration of hGH cDNAs into recombinant plasmids. The success of these reactions, and thus of our preferential cloning strategy, was monitored by Southern blotting [12] as shown in Fig.1. Transformation of

competent Escherichia coli strain RRI with this ligated material, resulted in 18 colonies. Dot blot screening of plasmid DNA isolated from the bacterial colonies, allowed us to identify putative hGH cDNA in three clones. Digestion with diagnostic restriction enzymes and partial DNA sequencing (data not shown), confirmed the presence of hGH-22K cDNA inserts in two of these clones (designated as pDRhGH22K) and of the hGH-20K cDNA in the third clone (designated as pDRhGH20K).

Subcloning and expression of the hGH cDNAs.

We then proceeded to subclone both hGH cDNAs from their AatII to XmaI sites, into the efficient eukaryotic expression vector pAVE2 [22] which already harbors 3' end untranslated, polyadenylation signal and 3' end processing sequences from the hGH gene. These cDNA copies were not full-length. They lacked the 5' and 3' untranslated regions, as well as the first six codons of the signal peptide. To complete both cDNAs and thus generate minigenes, we used DNA pieces derived from: i) the hPL-3 gene (from the cap site to the PstI site towards the 3' end of the first exon) and ii) the hPL-3 cDNA from this PstI site to an AatII site at the beginning of the second exon (codon -20 of signal peptide, which is 26 aminoacids long). In spite of the tailoring of these gene pieces, the mature hGH proteins derived from these hybrid minigenes are coded entirely by

the hGH cDNAs. The resulting hybrid plasmids, named pAVE2hGH22K and pAVE2hGH20K, are diagrammed in fig.2.

After transfection of COS-7 cells with the pAVE2 derivatives as well as with positive (pAVE1hGH) and negative (pAVE2) controls [22], total cellular RNAs were obtained and analyzed by Northern blot [18]. As shown in fig. 3, the transiently expressed hPL-hGH hybrid RNAs were specifically detected by hybridization with the ^{32}P-labeled hPL cDNA probe.

Cell culture production of hGH 22K and 20K.

To verify the correct expression of the transfected novel minigenes, the production of secreted hGH by the transfected COS-7 cells was determined by RIA. Values of secreted hGH-22K averaged 1259 ng per 60 mm culture dish of transfected COS-7 cells. The average value for secreted hGH-20K was 41 ng per the corresponding cultured dish.

As shown above in the Northern blot experiment, the hybridization signals given by the RNAs isolated from cells transfected with either pAVE2hGH22K or pAVE2hGH20K, are similar in intensity. This indicates that in our transfection assays, both minigenes are similarly efficient in generating mature mRNAs. In other studies it has been shown for the transiently expressed hGH gene, that the mRNA expression levels are directly reflected in hGH protein levels [23]. Thus, one explanation for having

such surprisingly low RIA values for hGH-20K, could be a poor recognition of this variant by the highly specific RIA. Similar results were found by Lewis et al. (1978) who provided evidence that hGH-20K was only one-third as active as the 22K hGH in their RIA [6].

To test this hypothesis, we decided to visualize both hGHs expressed and secreted into the medium used in the transfection assays. The proteins synthesized by the transfected cells were labeled with L-[^{35}S] methionine and those secreted into the culture medium were resolved by SDS-PAGE. The secreted proteins were then detected by autoradiography [18]. As it can be seen in fig. 4, the cells transfected with pAVE1hGH, which bears the hGH-N gene expressed hGH-22K abundantly; but, the 20K form derived from it was expressed at a lower proportion. The hGH-22K minigene behaved as the hGH-N gene directing the expression of large amounts of the 22K form of hGH, but in this case no hGH-20K was observed, as expected. On the other hand, the cells transfected with the hGH-20K minigene expressed the hGH-20K at levels above those found in cultures transfected with hGH-N gene, but below those of the hGH-22K produced by either of the other two recombinant plasmids. We still do not have an explanation for these differences in expression between the 22K and 20K hGH minigenes.

Adipogenic activity of the recombinant hGHs.

Further confirmation of the authenticity of secreted hGHs was obtained by evaluating their ability to specifically promote differentiation of 3T3-F442A preadipocytes cells to adipose cells [2]. Results obtained studying the biological activities of hGH variants, indicated that measurement of adipogenic activity is a much more sensitive assay for hGH activity than the measurement of body weight increase [24]. Control 3T3-F442A cell cultures supplemented with met(hGH)^{bac} (25 ng/ml) showed a 100% differentiation. Those cells supplemented with media samples from cultures transfected with pAVE2hGH22K also presented a significant proportion of differentiated cells (see table I). On the other hand, cultures exposed to medium containing hGH-20K secreted by transfected COS-7 cells, reached lower levels of adipose conversion (see table I). These results agree with the hGH-22K and hGH-20K protein levels detected by autoradiography (see above). In addition, they were similar to those obtained in experiments using pAVE1hGH as positive control (data not shown). The non-adipogenic medium from cells transfected only with the vector (pAVE2) did not stimulate this diferentiation process (table I).

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Figure 1. Southern blot analysis to confirm the preferential cloning of pituitary hGH cDNAs. DNA samples obtained during the cloning procedure of the hGH cDNAs were size fractioned, transferred to nylon membrane and hybridized with a radiolabeled hPL cDNA probe. Lane cDNAs: total pituitary cDNA; XmaI+AatII: total pituitary cDNA digested with AatII and XmaI; Cloning: ligation products of the AatII and XmaI-digested pituitary cDNA with similarly treated pUC19 vector; Vector: AatII and XmaI-digested pUC19 (negative control); Control: hPL cDNA (positive control) and M: 1kb Ladder. Left-hand= 1.4% agarose gel stained with ethidium bromide; right-hand= autoradiography.

Figure 2. Construction of pituitary growth hormone expression vectors. The plasmids pDRhGH20K in A and pDRhGH22K in B along with an intermediate plasmid (C) constructed in our laboratory containing a hPL-bGH-hGH hybrid minigene were used for the construction of pAVE2 derivatives vectors. A series of isolated DNA fragments (F,G,H and I) were assembled in different combinations in reactions catalyzed by T4 DNA ligase. The pAVE2hGH20K (D) was constructed with the fragment F from the coding sequence of 20K hGH cDNA (seventh codon of pre-hormone up till four nucleotides downstream termination codon) and the fragments H and I from pAVE2bGH, which lack the corresponding coding sequence of the hPL-bGH-hGH hybrid minigene. Like wise, pAVE2hGH22K (E) was constructed using fragment G instead of fragment F. Restriction sites with an asterisc are not unique in the maps shown. Stippled box, hGH cDNAs; open box, bGH cDNA; horizontally striped box, hPL-3 gene, vertically striped box, hPL-3 cDNA; inclined striped box, hGH gene 3' end. Sizes are not drawn at scale.

Figure 3. Northern analysis of hGH mRNA levels in COS-7 cells transfected with various hGH expression vectors. Ten μg of total RNA from transfected COS-7 cells were electrophoresed in a 1.2% denaturing phosphate-agarose gel and transferred to nitrocellulose membrane. The probe was the radiolabeled hPL cDNA. The individual expression vectors which were transfected are indicated at the top of the autoradiography. H y L represent lanes containing total DNA from human pituitary gland and rat liver, respectively. Lane labeled C represents total RNA of mock-transfected cells. For simplicity, we used pAVEhGH22K and pAVEhGH20K instead of pAVE2hGH22K and pAVE2hGH20K as the names of these recombinant plasmids.

Figure 4. Detection of the radiolabelled recombinant proteins encoded by hGH chimeric plasmids. Ten μg of DNA from the indicated expression vectors were transfected into COS-7 cells and the synthesized proteins were labelled with L-[^{35}S]methionine for 4 h. A 200 μg of protein sample was analyzed by electrophoresis on a 5-13% discontinuous polyacrylamide (SDS) gel. Gels were dried and subjected to autoradiography. The molecular weights of hGH recombinant proteins are indicated by arrows on the right.

Table I. Stimulation of adipose conversion by recombinant hGH's secreted by transfected COS-7 cells.

Plasmids	Adipose Conversion * (%)
None	0.0 ± 12.6
pAVE2	0.0 ± 1.7
pAVEhGH22K	100.0 ± 11.5
pAVEhGH20K	66.4 ± 4.5

* Values are average and standard deviations representing each data from three independent experiments.

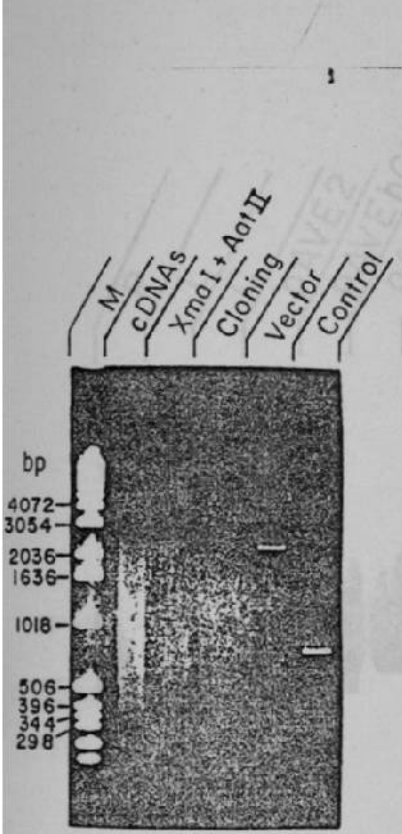

Figure 1

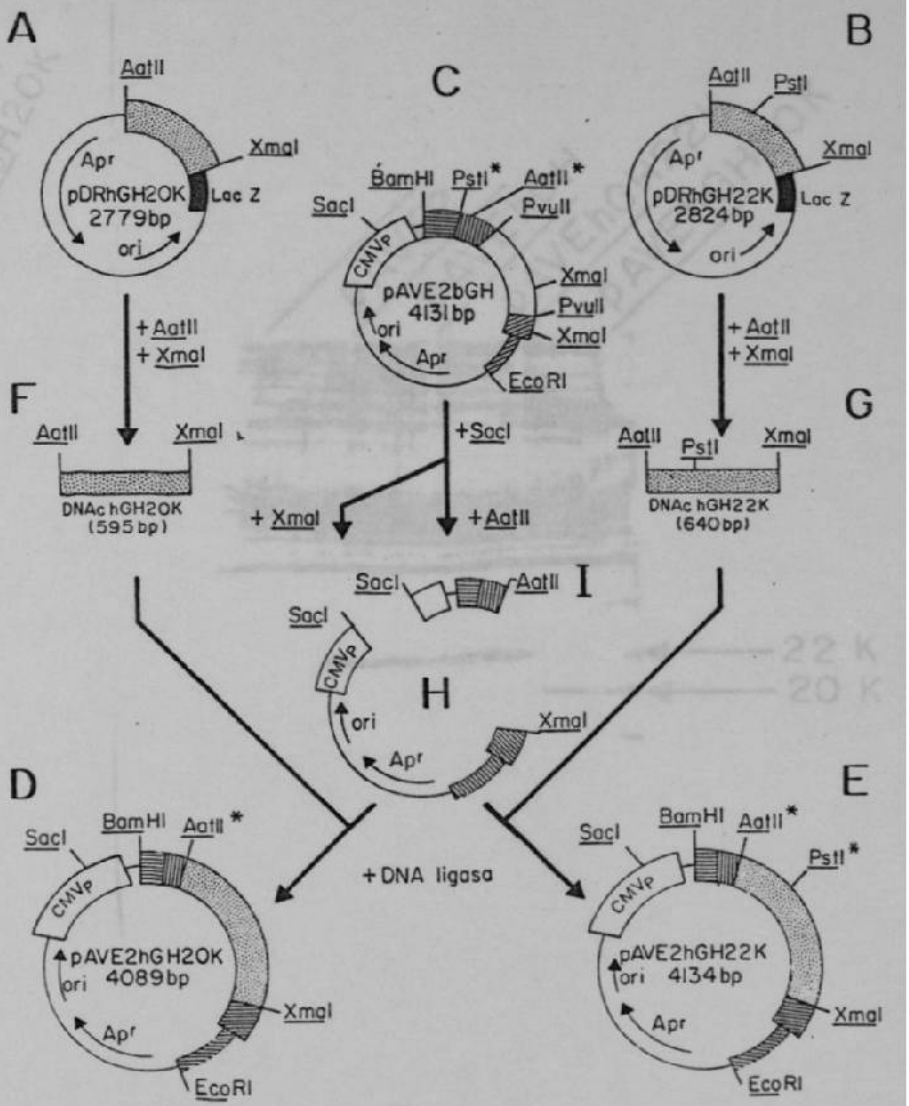

Figure 2