Synthesis of Growth Hormone-Prolactin Chimeric Proteins and Processing Mutants by the Exchange and Deletion of Genomic Exons*  

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To test the feasibility of synthesizing recombinant peptide hormones by exon deletion and exchange, we have constructed and expressed hybrid human growth hormone (hGH)-rat prolactin (rPrl) genes in which the third and fourth exons of the hGH gene are deleted and separately replaced by the corresponding exons of the rPrl gene. These exon deletion and exon exchange genes were inserted into an SV40 viral vector, packaged, and expressed following acute viral infection of monkey kidney cells. Expression of the hGH gene lacking the third exon (hGHD3) was not detectable at the level of protein production. However, replacement of the deleted third exon in the hGHD3 gene with exon 3 of the rPrl gene (hGHP3) resulted in the efficient synthesis of a secreted chimeric protein. When the fourth exon of the hGH gene was deleted (hGHD4), the encoded protein was found only in the cytosol despite signal sequence processing. Replacement of the deleted fourth exon in this hGHD4 gene with exon 4 of rPrl resulted in the synthesis and secretion of both a chimeric protein (hGHP4) as well as a larger protein corresponding in size to prehGHP4. These results suggest that 1) exon exchange among distantly related genes in the GH family may be used to produce high levels of chimeric GH-related proteins, and 2) regions internal to the hGH protein may be critical in establishing normal protein processing and secretion.

The growth hormone (GH3)-prolactin (Prl) family of peptide hormones in mammalian species includes GH, chorionic somatomammotropin, and Prl (1). In the mouse two additional Prl-related hormones, prolipherin and proliferin-like protein, have been described (2, 3). In humans another GH-related gene, hGH-variant, has been isolated (4) and expressed in vitro (5), although it has yet to be identified in vivo. These hormones are all encoded by a family of genes which have evolved in structure and function from a common ancestral precursor over the past 400 million years (6). The primary structure of each of the rat and human GH-Prl related hormones has been determined from either direct protein sequencing or from the sequence of cloned cDNAs

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‡ The abbreviations used are: GH, growth hormone; Prl, prolactin; h, human; r, rat, MEM, minimal essential medium; SDS, sodium dodecyl sulfate; kb, kilobase; bp, base pairs.

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(18) using a nick-translated \(^{32}P\)-labeled hGH cDNA (19) probe. Bacteriophage DNA was isolated from mini lysates (20) of plaque-purified, hybridizing clones and was analyzed by Southern blotting (21) with hybridization and washing conditions as previously described (22). Bacteriophage DNA was isolated for subcloning into plasmids as previously described (23).

Recombinant DNA Constructions—All restriction and modification enzymes and linkers were obtained from Bethesda Research Laboratories (BRL, Gaithersburg, MD) or New England Biolabs (NEBL, Beverly, MA) and used according to the manufacturer’s specifications.

For blunt-ended ligations staggered ends were filled in using DNA polymerase I, Klenow fragment (BRL) in the presence of the appropriate nucleoside triphosphates. To decrease background from vector self ligation, all vectors were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Ligations were carried out at \(4^\circ\)C for 18 h in 15 ml reaction volumes containing a 2:1 molar ratio of the modified GH gene fragment to vector DNA and 400 units of T4 ligase (NEBL) in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl\(_2\), 10 mM dithiothreitol, 1 mM spermidine, and 1 mM ATP. Ligated DNA was used to transform competent (24) Escherichia coli HB101 cells.

Ampicillin-resistant colonies were screened for the desired inserts by \(in\ situ\) hybridization (25) with nick-translated hGH or rPrl cDNA probes. Individual positive colonies were further characterized by restriction analysis of plasmid DNA isolated from clarified lysates of 1 ml overnight cultures. Large-scale preparations of plasmids were isolated and used to transfect cells essentially as described (5).

When a cytopathic effect was observed, usually 2 weeks after transfection, the cells were frozen and thawed three times, then stored at \(-70^\circ\)C. DNA from this mixed viral stock was carried forward by washing confluent CVlp cells three times with PBS, and overlaying serum-free and leucine-free MEM (Select-A) or 200 ml of media were immunoprecipitated, after which the resultant mixed viral stock and the infected cells, the monolayer was washed twice with PBS and overlaid with 1 ml of MEM containing 26% DAEAR--dextran (28), 2 \(\mu\)g of the gel-purified SV40-hGH fragment, and 0.2 \(\mu\)g of purified supercoiled tsA239 DNA (a gift from J. Alwine, University of Pennsylvania). The flask was rocked gently for 20 min at room temperature, the DNA solution was removed from the flask, cells were washed twice with PBS, and once with minimal essential medium (MEM) containing 2% fetal calf serum (Flow Labs, McLean, VA). Cells were incubated in 5% CO\(_2\) at 37°C. When a cytopathic effect was observed, usually 2 weeks after transfection, the cells were frozen and thawed three times, then stored at \(-70^\circ\)C. DNA from this mixed viral stock was carried forward by washing confluent CVlp cells three times with PBS, then overlaying them with 1 ml of the thawed viral stock plus 1 ml of serum-free MEM. These cells were incubated at 37°C with gentle rocking for 2 h. 12 ml of MEM with 2% FCS were then added, and the cells were maintained at this condition for an additional 23–36 h. When labeling with the infected cells, the monolayer was washed twice with PBS and overlaid with 3 ml of serum-free and leucine-free MEM (Select-A, Gibco Labs, N. Y.) containing 1 ml of \([^{3}H]\)leucine [1-4,5-\(\beta\)]leucine, 60 Ci/mm, New England Nuclear]. After 4 hr incubation at 37°C, the media was removed for analysis, and the cells were washed three times in PBS and lysed in 0.5 ml of lysis buffer (0.5 M Tris-HCl, pH 7.5, 0.15 mM NaCl, 1% Triton X-100, 0.5% aprotinin). The media and lysate were both clarified at 15,000 \(\times\) g for 10 min and stored at \(-20^\circ\)C until analysis.

Construction of hGH-rPrl Chimeric Genes by Exon Exchange—The replacement of the third and fourth exons of hGH with the corresponding exons of rPrl was accomplished in two steps (Fig. 2). First, exon 3 and exon 4, along with flanking intron sequences, were sequentially removed from pSVhGH and were replaced with an Xhol linker to create the two hGH exon deletion genes, hGHd3 or hGHd4. Exon 3 was removed by digesting hGH with SstI followed by a partial digestion with Xmal. The larger of the two fragments generated by this digestion was gel-purified, Xhol linkers were ligated to its ends, and the plasmid was recircularized. The Xhol site is 112 bp from the 3' border of exon 2 and 35 bp from the 5' border of exon 4 as determined by DNA sequence analysis (data not shown). To remove exon 4 of the hGH gene, hGH was digested with Xmal and BgIII and recircularized. This digestion removes both exon 4 and the 5' half of exon 5 from the hGH gene. The 5' half of exon 5 was then replaced in the proper orientation by opening the exon 4 and 5 deletion plasmid by Xmal digestion and ligating the Xmal fragment containing the 5' half of exon 5 and the adjacent intron D to the Xmal site. This was followed by ligation of Xhol linkers to the free ends and recircularization. The inserted Xhol site is 62 bp from the 3' border of exon 3 and 43 bp from the 5' border of exon 5 as determined by DNA sequence analysis (data not shown).

DNA Sequencing—DNA sequence analysis was performed on isolated, end-labeled DNA fragments by the method of Maxam and Gilbert (31) using previously described modifications (13).

RESULTS

Molecular Cloning of the hGH Gene and Its Expression in an SV40 Recombinant Vector—An hGH gene was isolated by screening 400,000 plaques of a Charon 4A library of normal human genomic DNA. The identity of the hGH clone was established by its characteristic restriction pattern after double digestion with EcoRI and BamH1 or Xhol and BamH1 (4).

The 2.6-kb EcoRI fragment of the hGH genomic clone containing the entire hGH gene as well as 497 bp of 5' flanking region and 540 bp of 3' flanking region (4) was subcloned into the previously described pBR322-SV40 late-region deletion vector behind the SV40 late-region promoter (5, 33). The SV40 genome in this vector, once separated from pBR322, can be efficiently packaged in an SV40 capsid when an additional 2700 bp (±20%) of foreign DNA is inserted into the late region. The transcriptional orientation of the inserted hGH gene with respect to SV40 was established by restriction mapping (Fig. 1). A clone containing hGH 3' to the SV40 late promoter in the late transcriptional orientation (pSvGH-L) was selected for further study because the late orientation had been previously reported to result in higher levels of hGH expression than the early orientation (5).

The SV40-hGH recombinant genome was packaged in SV40 viral coat proteins for high efficiency infection by transfecting it into CVlp cells along with DNA from tsA239, a temperature-sensitive SV40 helper virus. Fresh host cells were subsequently infected with the resultant mixed viral stock and labeled at 36 h after infection with \([^{3}H]\)leucine for 4 h. \(H\)-labeled proteins secreted into the media were immunoprecipitated using anti-hGH antisera and analyzed by electrophoresis on SDS-polyacrylamide gel (see Fig. 4, lane GH). A band at approximately 22 kDa appears in the media of SVhGH-infected cells that is not present in the media of the mock-infected cells. This band co-migrates with purified pituitary hGH (data not shown). A hGH specific radioimmunoassay detects an hGH concentration varying between 0.2 and 10 \(\mu\)g/ml of media in various infections (data not shown).
Ethidium bromide staining and are noted to the left of the 0.8% of each of the three components (pBR322, SV40, and hGH). The sites of the pBR322-SV40 late region deletion vector. Gel analysis of approximately equal size. With the hGH gene in the late transcriptional orientation of SV40, pSVhGH-e, BglII, and flanking regions was isolated and ligated into one of the two EcoRI sites of the pBR322-SV40 late region deletion vector. Gel analysis of EcoRI digests of the resultant recombinant confirmed the presence of each of the three components (pBR322, SV40, and hGH). The identity and size of each of these EcoRI fragments were visualized by ethidium bromide staining and are noted to the left of the 0.8% agarose gel. The transcriptional orientation of the hGH gene in relation to the early and late promoters of SV40 was determined by digesting the plasmids with KpnI and BglII. With the hGH gene in the early transcriptional orientation of SV40, pSVhGH-e, BglII, and KpnI are on opposite sides of the plasmid and release two fragments of approximately equal size. With the hGH gene in the late transcriptional orientation, pSVhGH-L, the BglII and KpnI sites are adjacent and release a small fragment from the plasmid. In the diagram, pBR322 is stippled, SV40 is a heavy line, and hGH is a fine line. The five exons of the hGH gene are shown as open boxes and are numbered. The origin (Ori) of replication of SV40 is marked by the solid circle, and five exons of the hGH gene are shown as open boxes and are numbered. The origin (Ori) of replication of SV40 is marked by the solid circle, and five exons of the hGH gene are shown as open boxes and are numbered.

The second step in the construction of the hGH-rPrl hybrid genes was the isolation of the third and fourth exons of rPrl along with flanking intron sequences and their insertion into the XhoI site of pSVhGHd3 and pSVhGHD4, respectively. Exon 3 of rPrl was isolated on a 220-bp fragment by digesting the previously described rPrl genomic subclone, rPrl HindIII A (13), with DdeI. This fragment contains exon 3 as well as 35 bp of flanking intron B and 77 bp of flanking intron C. XhoI linkers were added to the ends of this fragment and it was then inserted into the XhoI linearized pSVhGHd3. A new clone, pSVhGHP3, with the rPrl exon 3 inserted in the proper transcriptional orientation, was identified by restriction mapping. The position and orientation of the rPrl exon 3 insertion was confirmed by labeling the hGHP3 plasmid at the PstI site in the remaining portion of hGH intron C, 32 bp 3’ to the XhoI ligation site and sequencing in the 5’ direction into the PstI exon 3 insert (data not shown). Rat Prl exon 4 was isolated from the rPrl HindIII B genomic subclone (13) on a 491-bp MboII-AcoII restriction fragment containing 92 bp of intron C and 219 bp of intron D. After addition of XhoI linkers, this fragment was inserted into the XhoI site of pSVhGHd4 and a recombinant plasmid with exon 4 in the correct transcriptional orientation, pSVhGHP4, was identified by restriction analysis. This was confirmed by labeling the BglII site in hGH exon 5 and sequencing in a 5’ direction across the more 3’ XhoI ligation site into the rPrl exon (data not shown).

For final analysis, the original clone, pSVhGH, and the four derivative clones, pSVhGHd3, pSVhGHP3, pSVhGHD4, and pSVhGHP4 were digested with EcoRI and subjected to Southern blot analysis using an hGH cDNA probe. The ethidium bromide-stained gel (Fig. 3, panel A) demonstrates a 4.36-kb pBR322 band in each lane, a 2.81-kb SV40 band in each lane, and a variably-sized hGH hybridizing band which is specifically identified by its hybridization to the hGH cDNA probe (Fig. 3, panel B). The hGHP4 gene co-migrates with SV40 (panel A and B, lanes 5, 2.75 and 2.81 kb, respectively) but can be separated from SV40 by secondary digestion with XhoI. This digestion releases the 419-bp rPrl exon 4 insert and the two halves (the 5’ 1450-bp fragment and the 3’ 890-bp fragment) of the remaining hGH gene (panel A, lane 6), both of which hybridize to the hGH probe (panel B, lane 6).

Expression of the hGH Exon Deletion Genes and Exon Exchange Genes—CV1p cells were infected with packaged viral stocks which contain each of the hGH exon deletion and exon exchange genes; pSVhGHd3, pSVhGHP3, pSVhGHD4, and pSVhGHP4. At a time suitable for late SV40 expression (24-48 h after infection), the cells were labeled with [3H]leucine and both media and cytosol were analyzed for hGH expression by gel electrophoresis and autoradiography before and after immunoprecipitation with anti-hGH antisera (Figs. 4 and 5). The SVhGHd3 genome showed no evidence of protein expression when compared to mock-infected cells (Fig. 4, d3 and M lanes), despite numerous attempts at transfections with several independent SVhGHd3 viral stocks. Northern analysis of these cells demonstrated the presence of an hGH hybridizing mRNA band of the size expected for hGHd3 mRNA (data not shown). Insertion of the rPrl exon 3 into the SVhGHd3 genome, creating the SVhGHP3 genome, markedly alters this result. CV1p cells infected with the SVhGHP3 viral stock synthesize a 23-kDa protein which is present in both the cytosol and the media (Fig. 4, P3 lanes). This protein is approximately of the size predicted for the hGHP3 protein (21.5 kDa) and is immunoprecipitated with anti-hGH antisera. The slight difference in size from that predicted for hGHP3 was not further evaluated. The structure of the chimeric protein does not predict the addition of an N-linked glycosylation site which might otherwise account for this size difference. As the sizing of proteins on SDS-polyacrylamide gel electrophoresis is dependent upon a number of parameters in addition to molecular weight, such as protein-SDS interactions, differences between measured and predicted size do not necessarily reflect real differences in protein composition. The level of hGHP3 secreted by the infected cells into the media is equal to or higher than that synthesized by the CV1p cells expressing the native hGH gene (Fig. 4, GH lane).

The medium from [3H]leucine-labeled cells infected with the SVhGHd4 viral stock was identical to mock-infected controls and showed no evidence of expression of an hGH-related protein even after immunoprecipitation with an anti-hGH antisera (Fig. 4, d4 lanes). However, in contrast to the finding with SVhGHd5, immunoprecipitation of cytosol from the SVhGHd4-infected cells revealed a band of 15.8 kDa which is not present in mock-infected cells (Figs. 4 and 5). Several repetitions of this experiment, all in serum-free media,
FIG. 2. Scheme for the construction of the hGH-deletion and hGH-rPrl recombinant genes. For details see “Results.” The hGH gene is shown as a solid line with its exons as solid rectangles, while the rPrl gene is shown as a dotted line with its exons as stippled rectangles. The XhoI sites in the hGHD3 gene, hGHD4 gene, and flanking the two rPrl exon fragments are represented by a zigzag line. A 1-kb scale is shown in the lower right.

FIG. 3. Restriction endonuclease mapping of the hGHD3, hGHP3, hGHD4, and hGHP4 recombinant genes. Each of the hGH deletion or hGH-rPrl recombinant plasmids was digested with EcoRI (lanes 1–5) or EcoRI and XhoI (lane 6), fractionated by size on a 0.8% agarose gel, stained with ethidium bromide (panel A), and analyzed by Southern blotting using an hGH probe (panel B). The DNA samples are pSVhGH (lane 1), pSVhGHD3 (lane 2), pSVhGHP3 (lane 3), pSVhGHD4 (lane 4), and pSVhGHP4 (lanes 5 and 6). The positions of DNA size markers, λDNA digested with HindIII, are indicated to the left of the gel.

using short and long labeling times, failed to show evidence of hGHD4 secretion into the media. This result suggests that the hGHD4 gene encodes a hGH-related protein of about 15.8 kDa, the size predicted for hGHD4, but that this protein is localized to the cytosol and is not secreted into the media. The expression of hGHP4 was compared in parallel with that of hGHD4 and mock-infected control cells (Fig. 5). When compared to the mock-infected cells, immunoprecipitation of the labeled cytosol from hGHD4 infected cells demonstrates a protein of about 15.8 kDa (the same as in Fig. 4), and immunoprecipitation of the cytosol from the SVhGHP4 infected cells demonstrates a protein of 23.5 kDa. The calculated molecular weight of hGHP4 is 22,600. As in the case of hGHD4, hGHP4 is not seen in the media without immunoprecipitation (data not shown). However, in contrast to hGHD4, immunoprecipitation of the hGHP4 media demonstrates two faint bands of 23.5 and 25.2 kDa. The lower of the two bands co-migrates with that from the immunoprecipitated hGHP4 cytosol and is of the approximate size expected for mature secreted hGHP4. The larger immunoprecipitated protein is the size predicted for prehGHP4. The low level of

FIG. 4. Expression of the hGH, hGHD3, hGHP3, and hGHD4 genes. CV1 p cells were separately mock-infected (M) or infected with mixed viral stocks containing DNA from SVhGHD3 (d3), SVhGHD4 (d4), SVhGHP3 (P3), and SVhGH (GH). 36 h after infection the cells were labeled with [3H]leucine. At the end of the labeling period the media and clarified cytosol were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography without and with immunoprecipitation using anti-hGH antiserum as indicated in the figure. The position of protein molecular weight standards are indicated to the right of the gel.
with the cytosolic hGHd4 and hGHP4 proteins are marked. The position of the cytosolic hGHd4 and hGHP4 proteins are marked with arrowheads to the left of the gel.

expression of the secreted hGHP4, the presence of the larger secreted immunoreactive protein, and the lack of hGHd4 secretion suggest that the deletion or alteration of the fourth exon of hGH may adversely affect protein processing and secretion.

**DISCUSSION**

The addition or deletion of exons as carried out in vitro in the present study has precedent in the natural evolution of gene structure. Genes within multigene families can expand in functional diversity through the incorporation of new exons (34), either from related (35) or unrelated (36) donor genes or through alternate RNA splicing of exons in a single gene (37). Such exon flux can also be detrimental as demonstrated by the loss of gene function in several naturally occurring exon deletion mutants such as those found in the genes encoding collagen (38) or the LDL receptor (39). A number of recent studies have used in vitro exon exchange or exon deletion to map antigenic determinants (40). However, attempts to apply this approach to the study of eucaryotic protein processing and function have been limited to the study of the functional and immunologic determinants of the histocompatibility genes (40-42). The success of these latter studies may not by themselves predict the successful generalization of this approach to other genes and gene families because the members of the histocompatibility gene family are highly homologous and possess unusually well-defined relationships between their exons and the structural and functional domains in their encoded proteins. Therefore, it remains to be determined whether the exchange of exons between less homologous genes, lacking such a definitive relationship between exons and structural and functional protein domains, can reliably result in the synthesis of stable chimeric proteins.

In the present study the feasibility of synthesizing hybrid proteins containing exon-encoded regions originating from two structurally distinct peptide hormones was tested. The approach was to construct chimeric hGH genes which separately substituted exons 3 or 4 of the rPrl gene for the corresponding exons of the hGH gene (hGHP3 and hGHP4). A byproduct of the hybrid constructions was the generation of two hGH exon deletion genes; hGHd3 and hGHd4. Of the two exon deletion constructions and the two exon exchange constructions, three of these, pSVhGHP3, pSVhGHD4, and pSVhGHP4, were capable of expressing encoded hGH-derived proteins of approximately the predicted sizes. In the analysis of the three expressed hGH-derived gene constructions, the most striking finding is the high level of hGHP3 expression. The Prl and GH genes diverged approximately 400 million years ago and have been evolving on separate chromosomes for much of that time (6, 43-45). The primary sequences of the protein region encoded by exon 3 of the rPrl and hGH genes are only 25% homologous (4, 14, 32). Despite the substitution of this region of rPrl for that of hGH, the resultant hybrid protein, hGHP3, appears to be quite stable. This stability may be related to two conserved features of the secondary structure. First, the hydrophobicity/hydrophilicity profiles of exon 3 in hGH and rPrl are quite similar as predicted by computer analysis (data not shown) based upon the algorithms of Hopp and Woods (46). The exon 3-encoded protein regions from both the hGH and rPrl hormones are generally hydrophilic with a markedly prominent hydrophilic character in the last 10 residues. Secondly, the cysteines forming the major disulfide-bonded loop found in both hGH and rPrl are maintained in the hGHP3 hybrid protein. In hGH, this disulfide bond joins the cysteine at position 52, encoded by exon 3, to the cysteine at position 163, encoded by exon 5. In rPrl, this disulfide loop is similarly placed and joins the cysteine at position 56 in exon 3 and to the cysteine at position 172 in exon 5. Therefore, the major disulfide-bonded loop should be retained in the hGHP3 product, as well as the potential for retention of higher order structures and hydrophobic/hydrophilic interactions.

In repeated experiments we could not detect protein expression of pSVhGHD3, despite the presence of hGHd3 mRNA in the infected cells. The reason for this lack of hGHd3 protein expression remains unexplained. One possibility is that cysteine 56, encoded in exon 3, is lost, thereby disrupting the major disulfide loop and resulting in protein instability or protein denaturation and insolubility. Consistent with this is the finding that the insertion of the third exon of rPrl into the pSVhGHD3 construction restores this cysteine and in parallel restores expression of the gene to high levels. The lack of expression in the hGHd3 gene, similar to the loss of gene function in naturally occurring exon-deletion mutants (38-40), was not analyzed further in the present study.

Both the exon 4 deletion and the exon 4 exchange genes express protein products but each displays significant aberrancy. The hGHd4 protein appears to undergo normal signal peptide cleavage based upon observation of identically sized cytosolic hGHd4 and hGHD4 synthesized in vitro from the translation of hGHd4 mRNA in the presence of dog pancreas membranes (data not shown). However, hGHd4 is apparently not secreted as it can only be found in the cytosol. It is unlikely that its absence from the media is due to proteolytic degradation since hGHd4 is absent even when protein labeling...
is done in serum-free media. Therefore, we conclude that hGHd4 undergoes signal peptide cleavage but is not secreted. This is similar to the finding of Guan and Rose (47), who reported that a hybrid rat GH gene containing the transmembrane anchor region of the vesicular stomatitis G-protein was blocked in its transport to the cell surface at the level of the Golgi apparatus, despite normal signal peptide processing. hGHP4 is present in both media and cytosol. However, two abnormalities are observed. One is the low level of expressed protein compared to both hGH and hGHP3. The second is the appearance of a higher molecular weight 25,200 hGH compared to both hGH and hGHP3. The second is the high level of hGHP3 protein synthesis supports the suggested that these properties do not entirely be critical in export of hGH and that its deletion in hGHd4 might also be critical in export of hGHd4, its deletion in hGHd4 to maximally test this approach. The detailed description of this regulatory element.

The high level of hGHP3 protein synthesis suggests the feasibility of this exon exchange approach for studies of structural and functional relationships in the GH-Prl gene family. In the present study, this exchange was done between heterologous species to maximally test this approach. The demonstrated ability to synthesize a chimeric hGHP3 in large quantities suggests that similar recombinations between other members of the GH-Prl gene family, either inter- or intraspecies, may also be successful. The synthesis of such proteins will allow their isolation for functional analysis with the goal of mapping regions responsible for the diverse spectrum of activity displayed by these hormones. Close attention to, and investigation of, defects in gene expression in the exon deletion and exchange genes which are not well expressed, such as hGHd4 and hGHP4, may suggest new subtleties in those processes necessary for the expression of genes encoding secreted proteins.

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