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Expression of two human growth hormone genes in monkey cells infected by simian virus 40 recombinants

(recombinant DNA/gene transfer/growth factors/protein transport)

GEORGE N. PAVLAKIS*, NAOMI HIZUKA†, PHILLIP GORDEN†, PETER SEEBURG‡, AND DEAN H. HAMER*

*Laboratory of Biochemistry, National Cancer Institute, and †Diabetes Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205; and ‡Division of Molecular Biology, Genentech, Inc., South San Francisco, California 94080

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ABSTRACT **We have constructed simian virus 40 recombinants carrying two different human growth hormone (hGH) genes. Monkey kidney cells infected with these recombinants synthesize, process, and secrete hGH. The product of gene 1, which has coding sequences identical to those of a cloned hGH complementary DNA, is indistinguishable from pituitary hGH by several criteria. The product of gene 2, which is predicted to encode a variant protein, is less immunoreactive than pituitary hGH but binds efficiently to hGH cell surface receptors. These results show that gene 2 has the potential to be expressed into a previously unidentified form of hGH. They also demonstrate that it is possible to produce a mature hormone by gene transfer in eukaryotic cells and indicate the utility of the simian virus 40–monkey cell system for producing and characterizing secreted animal cell proteins.**

Human growth hormone (hGH) is synthesized by the acidophil cells of the anterior pituitary as a prehormone containing a hydrophobic amino-terminal signal sequence that is removed during secretion (1, 2). The mature hormone exhibits multiple biological effects *in vivo*, including diabetogenic, insulin-like, lactogenic, and growth-promoting activities (reviewed in ref. 3). Most hGH preparations can be separated into multiple bands by high-resolution isoelectric focusing or gel electrophoresis (refs. 4–6 and references therein). Some of these appear to result from posttranslational modification (7, 8) and others represent primary sequence variants. The best studied of these is the M_r 20,000 ("20K") variant, which is a single polypeptide chain identical to the major form of hGH except that it lacks amino acid residues 32–46 (9, 10). It is also known that hGH exists in heterogeneous forms in human plasma with respect to molecular size and biological properties (11–13). Whether these variants are important in clinical conditions in which hGH responsiveness is inappropriate for its plasma concentration is unknown, and there is no information as to whether the different forms could represent different gene products.

Recent DNA cloning experiments show that the human genome contains at least seven different hGH-related genes (14, 15). One of these, designated here as the hGH1 gene, has been shown by sequence analysis to be capable of encoding the predominant form of pituitary hGH (F. DeNoto, D. Moore, and H. Goodman, personal communication). A second gene, referred to as the hGH2 gene, is highly homologous to the hGH1 gene but contains 14 point mutations that are expected to lead to amino acid substitutions in the mature hormone (unpublished data). This suggests that some of the heterogeneity of hGH might be due to the expression of multiple closely related but nonidentical genes.

As a first step in testing this hypothesis we have inserted the hGH1 and hGH2 genes into a simian virus 40 (SV40) cloning vector and have used the resulting recombinants to infect primary cultures of monkey kidney cells. Previous experiments have demonstrated the utility of the SV40–monkey cell system for gene transfer and for the analysis of various steps in mRNA formation (reviewed in ref. 16). Here we have used this system to compare the synthesis, processing, and secretion of hGH1 and hGH2 and to produce sufficient amounts of these proteins for initial characterization.

MATERIALS AND METHODS

Procedures for the construction, propagation, and characterization of recombinant plasmids and viruses have been described (16, 17). The SV40 vector used to clone the hGH genes was derived from the viable deletion mutant *dl2005* (18), which has undergone a 250-base pair deletion in the viral early gene region. This allows us to insert approximately 2.7 kilobase pairs (kb) of foreign DNA into the late gene region as compared to 2.45 kb for the equivalent wild-type vector (unpublished results). Experiments with recombinant plasmids and viruses were carried out according to the National Institutes of Health guidelines.

To label proteins, a culture of 2×10^7 infected or uninfected cells was incubated either for 3 hr in 5 ml of medium containing [^{3}H]leucine [197 Ci/mmol, Amersham (1 Ci = 3.7×10^{10} becquerels)] at 200 μCi/ml as the only leucine or for 24 hr in 20 ml of medium containing 10% the normal level of leucine and [^{3}H]leucine at 20 μCi/ml. After the medium had been removed the cells were lysed in 1 ml of 0.5 M Tris·HCl (pH 7.5)/0.15 M NaCl/1% Triton X-100/0.5% aprotinin (Sigma). The media and cell extracts were treated with excess rabbit anti-hGH antibody (National Pituitary Agency) followed by protein-A-bearing *Staphylococcus aureus* (19). Immunoprecipitated proteins were collected by centrifugation, eluted by boiling 5 min in 60 mM Tris·HCl (pH 6.8)/1% $NaDodSO_4$/1% 2-mercaptoethanol, then analyzed by electrophoresis through a 20% acrylamide/$NaDodSO_4$ gel and fluorography (20, 21). Partial chymotrypsin digestions (22) of the immunoprecipitated proteins were performed in 50-μl reaction mixtures containing 20 μg of unlabeled pituitary hGH and 2.5 μg of chymotrypsin. Aliquots were removed and boiled after 5, 10, and 60 min of incubation at 37°C, pooled, and then analyzed by 20% acrylamide/$NaDodSO_4$ gel electrophoresis.

Double antibody radioimmunoassays with rabbit anti-hGH antibody were carried out as previously described (23), using hGH (preparation HS 2243E) and antibody from the National Pituitary Agency. Radioimmunoassays with guinea pig anti-hGH antibody were performed by Hazleton Laboratories (Vi-

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Abbreviations: hGH, human growth hormone; SV40, simian virus 40; kb, kilobase pair(s).

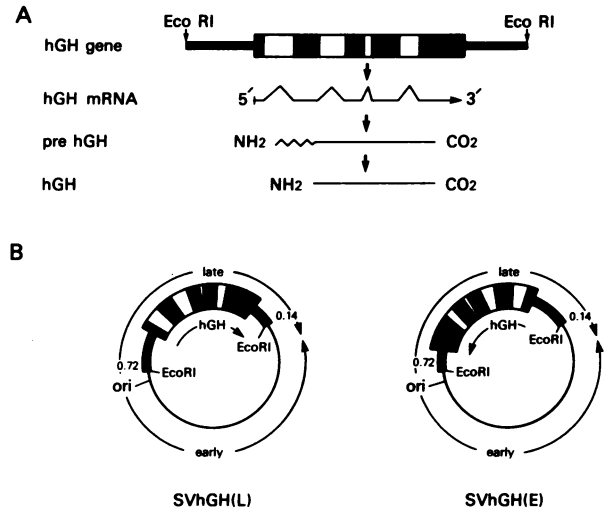

FIG. 1. SV40–hGH recombinants. (*A*) Both hGH genes were cloned on 2.7-kb *Eco*RI fragments containing five structural sequences (solid bars) and four intervening sequences (hollow bars) together with 500 base pairs of 5′ flanking sequences and 550 base pairs of 3′ flanking sequences (thick lines) (14). Translation of hGH mRNA yields pre-hGH containing an amino-terminal signal sequence of 23 amino acids. This is processed to generate mature hGH. (*B*) The two hGH genes were inserted, in both possible orientations, into an SV40*dl2005* vector (thin lines) extending clockwise from the *Bam*HI site at 0.14 map unit to the *Hpa* II site at 0.72 map unit. Both vector sites were converted to *Eco*RI sites by using oligonucleotide linkers (unpublished results). *ori*, Origin of replication.

enna, VA). Radioreceptor assays using either IM-9 cultured human lymphocytes (24) or pregnant rabbit liver membranes were performed by a modification of the method of Tsushima and Friesen (25). Our modification involved use of 50 mM Hepes buffer and an incubation at 4°C for 16 hr.

RESULTS

SV40–hGH Recombinants. The hGH1 and hGH2 genes were originally cloned in phage λ as 2.7-kb *Eco*RI fragments of human placental DNA (14). The two genes are approximately 95% homologous but can be readily distinguished from one another by the fact that gene 1 contains one *Bam*HI site whereas gene 2 contains two sites. DNA sequence analysis shows that both the gene 1 and gene 2 *Eco*RI fragments contain approximately 500 base pairs of 5′ flanking sequences and 550 base pairs of 3′ flanking sequences as well as five hGH structural sequences (exons) separated by four intervening sequences (introns) (Fig. 1*A*). The coding sequences of hGH1 are identical to those in cloned hGH complementary DNA, suggesting that this gene is expressed into the major form of pituitary hGH (F. DeNoto, D. Moore, and H. Goodman, personal communication). In contrast, the hGH2 gene differs from the cDNA by several base changes, 14 of which are expected to lead to amino acid substitutions in the mature hormone (unpublished data).

The two different 2.7-kb hGH gene fragments were inserted, in both possible orientations, into a SV40 vector that retains the origin of viral DNA replication, a functional early gene region, and the extreme 5′ and 3′ termini of the late gene region (Fig. 1*B*). In the SVhGH(L) recombinants the hGH1 and hGH2 genes are in the same orientation as SV40 late gene transcription, whereas in the SVhGH(E) recombinants they are in the

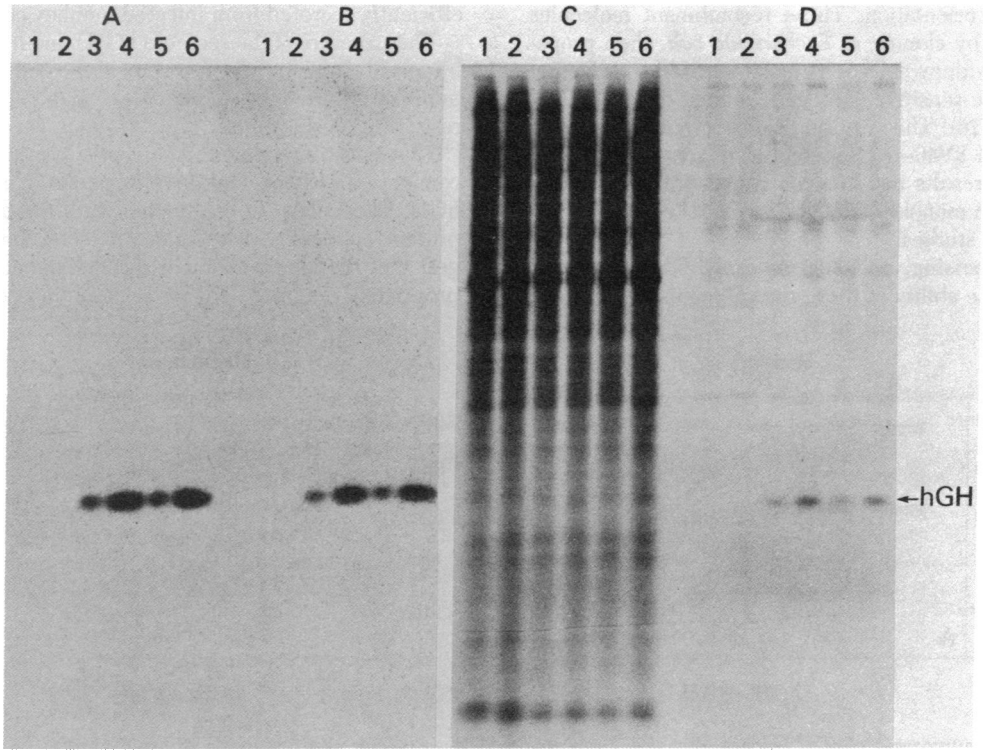

FIG. 2. Synthesis of hGH in infected monkey cells. Monolayers of 2×10^7 monkey kidney cells were labeled with [^{3}H]leucine (200 μCi/ml) at 40–43 hr after infection. Cellular and media proteins were analyzed by immunoprecipitation with excess anti-hGH antibody and 20% acrylamide/$NaDodSO_4$ electrophoresis. (*A*) Immunoprecipitates of cell extracts from 5×10^6 cells. (*B*) Immunoprecipitates of media from 10^6 cells. (*C*) Total cellular extracts from 10^5 cells. (*D*) Total media from 10^5 cells. In each panel the lanes represent cells infected with: 1, no virus; 2, wild-type SV40; 3, SVhGH1(E) plus helper; 4, SVhGH1(L) plus helper; 5, SVhGH2(E) plus helper; 6, SVhGH2(L) plus helper. The symbols (E) and (L) refer to the orientation of the genes relative to the late promoter of the SV40 vector.

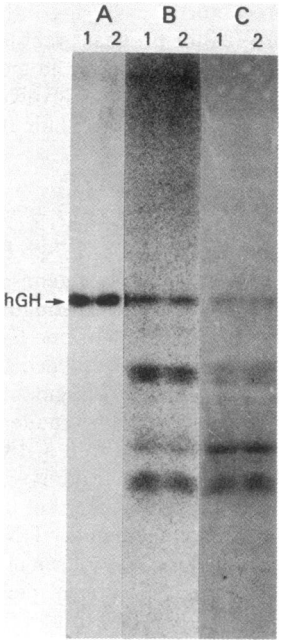

FIG. 3. Partial chymotrypsin digests. [^{3}H]Leucine-labeled hGH1 and hGH2 were purified by immunoprecipitation of the media from cells infected with SVhGH1(L) or SVhGH2(L). The precipitated proteins were eluted by boiling in $NaDodSO_4$, mixed with 20 μg of unlabeled pituitary hGH, partially digested with chymotrypsin, and analyzed by 20% acrylamide/$NaDodSO_4$ gel electrophoresis. (*A*) Fluorogram of undigested samples. (*B*) Fluorogram of chymotrypsin-digested samples. (*C*) Photograph of panel B stained with Coomassie brilliant blue. In each panel lane 1 represents hGH1 and lane 2 represents hGH2.

opposite or early orientation. These recombinant molecules were constructed by cloning in *Escherichia coli*, then propagated in monkey kidney cells as virions by mixed transfection with a temperature-sensitive early gene mutant of SV40 (SV40 tsA_{239}) as helper (26). The resulting stocks of virus contained approximately 10% SV40–hGH recombinant genomes and 90% helper genomes (results not shown). These viral stocks were used to infect fresh monolayers of primary monkey kidney cells for all subsequent studies.

Synthesis, Processing, and Secretion of hGH in Infected Monkey Cells. The ability of these recombinants to direct the synthesis of hGH was tested by labeling infected cells with [^{3}H]leucine and analyzing the cellular proteins by immunoprecipitation and acrylamide/$NaDodSO_4$ gel electrophoresis. As shown in Fig. 2*A*, cells infected with each of the four recombinants synthesized a protein that comigrated with authentic pituitary hGH and was absent from uninfected and wild type SV40-infected controls. The amount of this protein synthesized was similar for SVhGH1(L) compared to SVhGH2(L) and for SVhGH1(E) compared to SVhGH2(E); in both cases about 3-fold more polypeptide was made in the late (L) than the early (E) orientation. Thus the two genes function equally well, but the level of expression depends upon their orientation relative to the vector.

To determine whether the hGH was secreted from monkey cells, we repeated the immunoprecipitation and gel analysis on the media from the control and recombinant-infected cells. Fig. 2*B* shows that both the hGH1 and hGH2 proteins are present in the media. Quantitation of the gel lanes by microdensitometry showed that the secreted material represents approximately 80% of the total hGH synthesized in a 3-hr pulse with [^{3}H]leucine. To exclude the possibility of cell leakiness or lysis, as compared to active transport, we also analyzed the total intracellular and media proteins without immunoprecipitation. The cell extract (Fig. 2*C*) showed a complex array of bands, as expected from the fact that SV40 does not shut down host cell synthesis, and it was not possible to resolve hGH from the background of cellular proteins. In contrast, the media (Fig. 2*D*) contained a much more discrete set of proteins, and hGH could readily be visualized as a predominant band in the recombinant-infected samples but not in the controls. No SV40 capsid proteins were detected in the media, indicating that cell lysis was not occurring at the time of labeling (40 hr after infection). These results show that both hGH1 and hGH2 are specifically and efficiently secreted from infected monkey cells.

The structure of the secreted hGH1 and hGH2 was analyzed by partial chymotrypsin digestion of the $NaDodSO_4$-denatured proteins followed by acrylamide/$NaDodSO_4$ gel electrophoresis. Fig. 3 shows that both proteins gave rise to identical [^{3}H]leucine-containing chymotryptic peptides and that these comigrated with the peptides obtained from unlabeled pituitary hGH. These data, in conjunction with the fact that the intact proteins comigrate with pituitary hGH on $NaDodSO_4$ gels, suggest that the amino-terminal signal sequences have been appropriately removed. The hGH1 protein also comigrated with

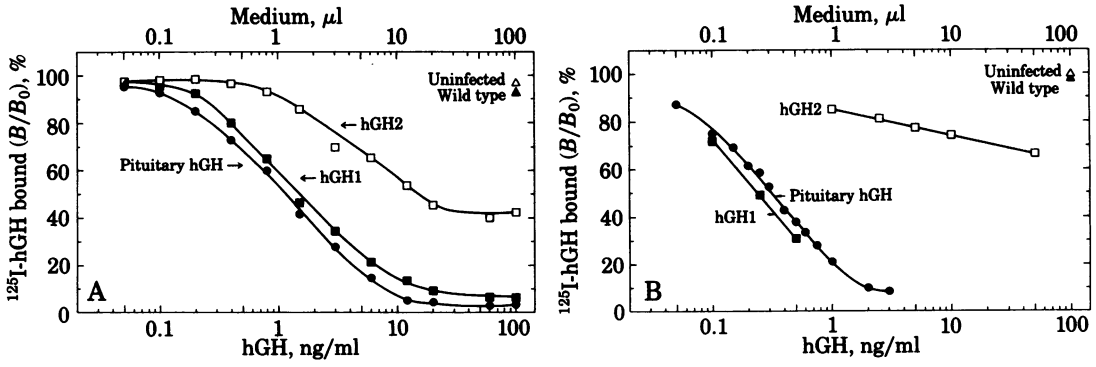

FIG. 4. Radioimmunoassay of hGH preparations. In *A* a rabbit anti-hGH serum was used and in *B* a guinea pig anti-hGH serum was used. For the assay shown in *B* the standard and ^{125}I-labeled hGH (^{125}I-hGH, specific activity $\approx$30 μCi/μg) were the same as used in the radioreceptor assays shown in Fig. 5. The media used in this experiment were collected from approximately 2×10^7 cells that had been infected with SVhGH1(L) or SVhGH2(L) and labeled in 20 ml of medium containing [^{3}H]leucine at 20 μCi/ml from 24 to 48 hr after infection. Analysis of these samples by acrylamide/$NaDodSO_4$ gel electrophoresis and fluorography showed that they contained approximately equal amounts of [^{3}H]leucine-labeled hGH. The lower scale refers to the concentration of pituitary hGH standard added to the assay and the upper scale refers to the amount of infected cell medium added. The total sample volume was 100 μl in all assays. Controls of medium from uninfected cells and cells infected with wild-type SV40 are shown. *B*, ^{125}I-hGH bound; B_0 ^{125}I-hGH bound in the absence of competitor.

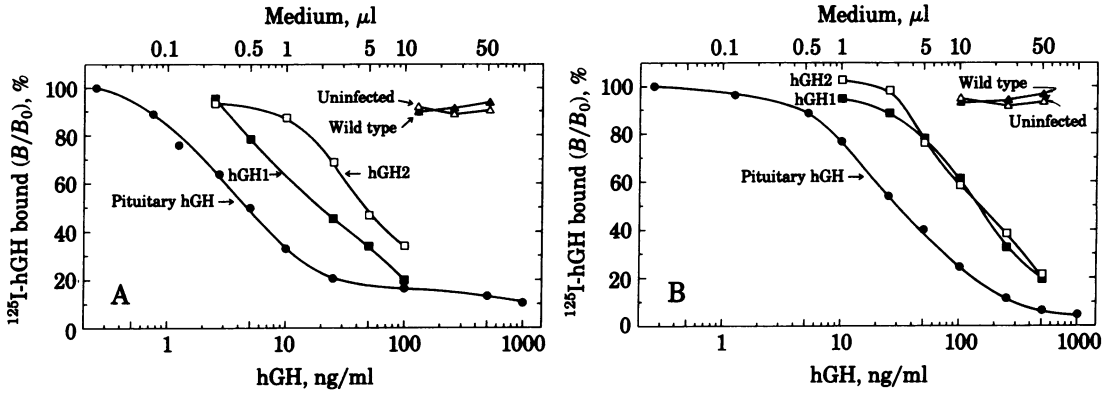

FIG. 5. Radioreceptor assay of hGH preparations. In *A* data from the IM-9 cultured human lymphocyte assay are shown and in *B* data from the pregnant rabbit liver membrane assay are shown. In each instance the assay contained the receptor preparation, ^{125}I-hGH, and various amounts of the unknown hGH preparation. These ingredients were diluted in buffer to final volume of 500 μl and the incubation and separation of the bound and free components were carried out as described (24, 25). The media were the same samples used in Fig. 4. Note that the relationship of the lower and upper scales is different from that in Fig. 4.

pituitary hGH in isoelectric focusing (27) and nonequilibrium pH gradient electrophoresis gels (28), but hGH2 could not be resolved in either system (results not shown).

Binding of hGH1 and hGH2 to Antibodies and Receptors. Fig. 4 shows double antibody radioimmunoassays of the media from SVhGH1- and SVhGH2-infected cells using either rabbit (Fig. 4*A*) or guinea pig (Fig. 4*B*) anti-hGH antibody. In order to ensure that we compared equal quantities of hGH1 and hGH2 polypeptides, the media used in this experiment were collected from cells uniformly labeled with [^{3}H]leucine. Scans of the NaDodSO$_4$ gel fluorograms showed that the SVhGH1 and SVhGH2 samples contained equal amounts of hGH polypeptide. The dose–response curves for hGH1 were parallel to the pituitary hGH standard in both assays. In contrast, hGH2 gave nonparallel curves with both antisera and it reacted less with the guinea pig antibody than with the rabbit antibody. Because of the nonidentical crossreactivity the most dilute samples of hGH2 give the highest values in radioimmunoassay. Under these conditions hGH2 had approximately 10% the immunoactivity of hGH1 with the rabbit antibody and less than 5% with the guinea pig antibody.

The ability of hGH1 and hGH2 to bind to hGH cell surface receptors was tested by radioreceptor assays using either the human lymphocyte line IM-9 (24) or pregnant rabbit liver membranes (25) as the receptor sources. In both systems hGH1 was indistinguishable from pituitary hGH (Fig. 5). Surprisingly, hGH2 was 50% as active as hGH1 in the lymphocyte assay and 100% as active in the liver membrane assay and gave dose–response curves parallel to the standard in both systems. Thus the ratio of receptor to immunoassay activity is approximately 1 for hGH1, whereas it is 10 or greater for hGH2 (Table 1). The identity of the hGH1 and hGH2 preparations in the liver assay is in contrast to the behavior of the M_r 20,000 hGH variant, which is only 3–20% as potent as pituitary hGH in this system (29).

Table 1. Relationship of receptor binding activity (radioreceptor assay; RRA) to immunoreactivity (radioimmunoassay; RIA) of the two different gene products

Assay system	RRA/RIA	
	hGH1	hGH2
IM-9 cultured human lymphocytes	0.87	≥10
Pregnant rabbit liver membrane	0.86	≥20

The RIA potency of hGH2 was calculated from Fig. 4*B* at the greatest dilution that exhibited competition. Because of the different slope of the curve, this represents a maximum estimate. Accordingly, the RRA/RIA ratios calculated for hGH2 are minimum values.

DISCUSSION

We have inserted two different hGH genes into SV40 and have shown that both genes can be efficiently expressed in infected monkey kidney cells. The hGH1 protein, as predicted from the DNA sequence, appears identical in all respects to the major form of pituitary hGH. In contrast, the hGH2 protein differs from authentic hGH both in its behavior on isoelectric focusing gels and in its low immunoreactivity, yet it binds to hGH receptors quite efficiently. These results show that, at least in the heterologous SV40–monkey cell system, gene 2 can be expressed into a novel variant form of hGH. Whether or not the gene 2 product has biological activity *in vivo* is unknown; we refer to it as a "growth hormone" simply because of its homology to pituitary hGH and its ability to bind to hGH receptors.

Both hGH1 and hGH2 are processed and secreted from monkey cells. Appropriate removal of the hydrophobic leader sequence was not unexpected, because the necessary enzymes are present in a variety of cell types and species (30). Also, Gruss and Khoury (31) have presented evidence that preproinsulin is processed to proinsulin in monkey cells infected with an SV40–insulin gene recombinant; however, the mature hormone was not produced due to failure to remove the internal C peptide. In pituitary cells hGH is sequestered into secretory granules prior to release into the bloodstream. The specific and efficient secretion of hGH from monkey kidney cells, which lack these granules, suggests that they are not essential for transport across the cell membrane.

We do not know to what extent transcription of the hGH genes depends upon their own promoters as compared to adjacent viral promoters, but it is interesting to note that the amount of protein produced in the late orientation is only about 3-fold greater than that in the early orientation. If transcription were completely dependent upon the SV40 promoters we would expect a ratio closer to 25:1 (unpublished results) because the later promoter is more active than the early promoter late in infection and because the early transcription unit is separated from the hGH genes by a polyadenylylation site.

Because both hGH genes are interrupted by four intervening sequences it is clear that their signals for RNA splicing must be

functional in our system. Recently, Lewis and coworkers (9, 10) have described a M_r 20,000 variant of hGH that lacks amino acids 32–46, and it has been suggested that this protein might result from alternative splicing of hGH mRNA (32). We have not been able to detect this protein in SVhGH-infected monkey cells. Therefore, it appears either that these cells utilize different RNA processing pathways than pituitary cells do or that the M_r 20,000 variant is the product of yet another hGH gene.

Both pituitary and plasma hGH consist of heterogeneous forms. For the most part this heterogeneity relates to higher molecular weight forms as exhibited by gel filtration of pituitary or plasma preparations (11, 12). The present genetic data support the idea that these higher molecular weight components represent posttranslational modifications of the molecule rather than different gene products. On the other hand, there are some clinical situations that could, in principle, be explained by the existence of primary sequence variants of hGH such as hGH2. In acromegalic patients it has been found that the major hGH component of plasma ("little hGH") has a higher receptor to immunoassay ratio than does the material purified from normal serum (13). It is apparent from Table 1 that this could be accounted for by the presence of hGH2 in the acromegalic serum. Thus, an increase in an hGH form similar to hGH2 would produce a greater bioactive effect (as predicted from receptor activity) than would be predicted from the immunoreactive hGH concentration.

Although the potency of an hGH preparation in the IM-9 cultured lymphocyte radioreceptor assay correlates well with the bioactivity of the preparation as measured in the classic rat bioassay, other circumstances could exist *in vivo* (33). For instance, a genetic variant of insulin has been described in a patient exhibiting insulin resistance (34). In this case the abnormal insulin molecule has decreased bioactivity but, more important, it acts as a partial agonist at the level of the insulin receptor and results in a shift in the insulin dose–response to the right. Whether a similar situation exists for variant short stature, a condition in which appropriate plasma concentrations of immunoreactive hGH fail to produce an appropriate tissue response (35–37), is presently unknown.

Radioimmunoassays (Fig. 4) show that SVhGH-infected monkey kidney cells produce as much as 50 μg per 2×10^7 cells per day (8×10^7 molecules per cell per day) of hGH, a level that compares favorably with cultured pituitary cells. Furthermore, because the monkey cells export only a small fraction of their own proteins, the hGH can be collected from the media in highly enriched form. The ability to produce milligram quantities of hGH2 should facilitate the search for this protein in human tissues and sera and will also allow us to test the biological activity of the variant protein by appropriate bioassays and animal tests. The high level of hGH production in our experiments suggests that the SV40–monkey system will be of general utility for characterizing secreted animal proteins, including those for which the gene is available but the function is unknown.

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