Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3644–3648, June 1985
Biochemistry

Expression of human choriogonadotropin in monkey cells using a single simian virus 40 vector

(RNA splicing/transfection/bioassay/glycoprotein hormone)

V. B. REDDY*, A. K. BECK*, A. J. GARRAMONE*, V. VELLUCCI*, J. LUSTBADER†, AND EDWARD G. BERNSTINE*

*Integrated Genetics, Inc., 31 New York Avenue, Framingham, MA 01701; and †Department of Medicine, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032

Communicated by Seymour Lieberman, February 20, 1985

ABSTRACT **We have inserted the cDNAs coding for both polypeptide subunits, α and β, of human choriogonadotropin (hCG) into a single simian virus 40 expression vector in such a way that they replace the viral VP2 and VP1 coding sequences, respectively. Monkey cells infected with this virus and the appropriate helper virus produce dimeric hCG. hCG produced in this system was shown to chromatograph identically to standard hCG preparations on gel filtration and to be biologically active in the mouse uterine weight assay.**

The three glycoprotein hormones lutropin (LH), follitropin (FSH), and choriogonadotropin (CG) act on the ovary to stimulate steroid synthesis and secretion. LH and FSH are secreted by the pituitary and together play a central role in regulating the menstrual cycle and ovulation. Human CG (hCG) is secreted by the developing placenta during the first trimester of pregnancy and is active in maintaining luteal function.

Each of the gonadotropins is a dimer consisting of two distinct polypeptide chains (1, 2) an α subunit and a β subunit. While the β chain of each hormone is unique, the same α chain is found in all three gonadotropins. The α and β subunits associate noncovalently to form the dimeric polypeptides. In the case of hCG, the α and β hCG subunits are 92 and 145 amino acids long, respectively. Glycosylation is a key feature of the gonadotropins; hCG is ≈30% carbohydrate by weight. The structure of its carbohydrates has been determined (3, 4). The common α subunit in hCG is glycosylated at two asparagine residues, while the hCG β chain is glycosylated at two asparagines and four serines (2). Although definitive data are not available, there is evidence that the carbohydrate portions of hCG are involved in its stability in the circulation (2, 5).

Simian virus 40 (SV40) has been used as a vector for the expression of a number of eukaryotic genes in monkey kidney cells (6–11). All the SV40 vectors that have been used can be grouped into two categories: late and early replacement vectors. The late replacement vectors make use of the late promoter, splicing, and polyadenylylation signals and are introduced into CV-1 cells along with helper SV40 DNA containing a temperature-sensitive (*tsA*) early gene (6, 7). The early replacement vectors, on the other hand, make use of the early promoter and early processing signals. They are introduced into Cos cells (8, 11) without any helper SV40 DNA because the integrated SV40 early region in Cos cells provides the missing early protein essential for SV40 DNA replication (12). Although SV40 has become a common expression vector, not more than one foreign gene at a time has been expressed by genetically engineered viruses. This report describes the construction of a late replacement vector that takes advantage of the splicing patterns of the SV40 late transcripts to express both hCG subunits in one cell. Use of this vector has allowed the production of biologically active hCG.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Viral DNA (form I) prepared from SV40 strain 776 was used in all constructions. pBR322 was used for cloning and amplification of DNAs in *Escherichia coli* strain HB101. Restriction endonucleases and DNA modifying enzymes were obtained from commercial sources. Radiolabeled compounds were obtained from New England Nuclear. CV-1 cells were kindly provided by P. K. Ghosh (Yale University). Antisera against α and β hCG subunits were obtained from Judith Vaitukaitis (Boston City Hospital). Restriction enzyme digestions, ligations, bacterial transformations, and sequence analysis were performed according to standard procedures. Each construction was confirmed by multiple restriction enzyme digestions and partial sequence analyses. Cloning and propagation of DNAs and viruses were performed according to the National Institutes of Health guidelines.

Cloning and Engineering of α and β hCG cDNAs. α and β hCG cDNAs were obtained by screening human placental cDNA libraries with complementary DNA probes. Sequence analyses confirmed the results reported earlier by Fiddes and Goodman (13, 14). The α hCG cDNA, which was ≈700 base pairs (bp) long, was cut at its unique *Nco* I site, which includes the initiator ATG codon, blunt-ended by filling in with the Klenow fragment of DNA polymerase I, ligated to *Hin*dIII linkers, and cloned into pBR322. The β hCG cDNA, which was ≈580 bp long, was cloned into pBR322 by means of *Bam*HI linkers. The plasmid was later restricted with *Hga* I, which cuts 2 bp 5′ to the ATG, filled in by Klenow polymerase, ligated to *Eco*RI linkers, cut with *Eco*RI and *Bam*HI, and cloned into pBR322 to obtain pβR/B.

Construction of the Expression Vector. The various steps leading to the final expression vector, pαβSVVPI, are illustrated in Fig. 1 and described in detail below.

pSVHR. SV40 DNA (form I) was linearized by partial *Hin*dIII endonuclease digestion, treated with Klenow polymerase in the presence of dNTPs, ligated to *Eco*RI and *Bam*HI, and cloned into pBR322. The plasmid pSVHR, which contains SV40 DNA between 0.95 map units (m.u.) and 0.14 m.u., including the origin of replication and the entire early region, was identified by restriction mapping.

pβSVVPI. pβR/B was methylated by using *Eco*RI methylase, restricted with *Nde* I, and *Eco*RI linkers were added to the ends after filling in with Klenow polymerase.

Abbreviations: bp, base pair(s); kb, kilobase(s); m.u., map unit(s); SV40, simian virus 40; hCG, human choriogonadotropin.

The plasmid was then cut with *Eco*RI and *Bam*HI and the fragments were ligated to *Eco*RI- and *Bam*HI-digested pSVHR. The ligation mixture was cut with *Sal* I to avoid reformation of pSVHR. Transformation of HB101 and screening of the resulting colonies with appropriate restriction endonucleases yielded the plasmid pβSVVPI, which was then cut with *Eco*RI, ligated, cut with *Bam*HI, and cloned into PBR322 to yield pβSVVPI Bam.

pSV266k. SV40 viral DNA was restricted with *Ava* II and the overhanging ends were filled with dNTPs and Klenow polymerase. The 682-bp-long SV40 fragment between 0.64 m.u. and 0.77 m.u. was isolated, ligated to *Hin*dIII linkers, and cut with *Hin*dIII and *Kpn* I. The resulting fragments were cloned into a pBR322 derivative (obtained by converting the original *Eco*RI site to a *Kpn* I site), and the plasmid containing the 266-bp SV40 DNA fragment (0.71 m.u. to 0.77 m.u.) was isolated.

pαβSVVPI. pβSVVPI Bam was cut with *Hin*dIII and *Bam*HI and the hCG-containing fragment was cloned into PSV266K. The resulting plasmid, pβVPI266K, was cut with *Hin*dIII and the α hCG cDNA was inserted as a 574-bp *Hin*dIII fragment. The plasmid pαβVPI266K was restricted with *Kpn* I and *Sal* I and cloned into pβSVVPI Bam to obtain pαβSVVPI (see Fig. 1).

Propagation of αβSVVPI in CV-1 Cells. pαβSVVPI was cut with *Bam*HI and ligated under conditions that promote self-ligation. At the same time, ptsA58 *Bam* (tsA58 SV40 viral DNA cloned into the *Bam*HI site of pBR322) was cut with *Bam*HI and self-ligated to give circular molecules. The DNAs were precipitated with ethanol and dissolved in sterile water. Approximately 1 μg of ptsA58 and 10 μg of pαβSVVPI DNAs were mixed with 2 ml of TBS buffer (15) and 1 ml of DEAE-dextran solution (2 mg/ml) and added to a monolayer of confluent CV-1 cells (in a T-75 flask) that had been washed twice with 10 ml of TBS buffer (16). After incubation at 37°C for 1–2 hr with occasional shaking, the cells were washed twice with TBS buffer, fed with 10 ml of Dulbecco's modified Eagle's medium containing 5% fetal calf serum, and left at 40°C for 10–15 days. After complete cell lysis, the medium, which served as a virus stock, was transferred to a test tube, frozen and thawed four times, and used to infect fresh CV-1 cells at 40°C.

Analysis of RNA. CV-1 cells were grown to confluence in roller bottles and infected with the recombinant virus stock. Five days after infection, the cells were trypsinized and pelleted by centrifugation. Cytoplasmic RNA was extracted by Penmans' procedure (17). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography, electrophoresed on formaldehyde/agarose gels (18), and transferred to nitrocellulose sheets. The RNA was hybridized with ^{32}P-labeled α and β hCG cDNAs.

Detection and Characterization of hCG Produced in CV-1 Cells. The hCG produced by infected CV-1 cells was detected immunochemically by using two commercially available assays. The first was the subunit-specific Beta III hCG radioimmunoassay produced by Serono Laboratories (Randolph, MA). The second was a dimer-specific enzyme-linked immunoassay (Tandem-E HCG) obtained from Hybritech (San Diego, CA). All assays were performed in duplicate according to the instructions provided by the suppliers.

Gel permeation chromatography of tissue culture supernatants was carried out on a column (1.2 × 95 cm) of Sephadex G-100 in 0.1 M ammonium acetate (pH 4.5). Fractions of 3 ml were collected. The column was calibrated by determining the position of elution of bovine serum albumin, ^{125}I-labeled hCG reference standard, ribonuclease, and [^{14}C]glucose.

Samples of each fraction were assayed for protein by measuring the absorbance at 280 nm; for the presence of β subunit by using a radioimmunoassay whose monoclonal antibody component recognizes both dimeric hCG and free β subunit; for dimeric hCG by using a monoclonal antibody recognizing only this molecular form; for α subunit with a polyclonal (rabbit) antiserum that recognized both free α and hCG dimer; and for *in vitro* biological activity by using a commercially available radioreceptor assay (Biocept-G; Wampole Laboratories, Cranbury, NJ).

The biological activity of the hCG produced by the CV-1 cells was determined *in vivo* by the mouse uterine weight assay (19). Two milliliters of tissue culture supernatants was injected (i.p.) into a series of C57BL/6J female mice, between 19 and 22 days old, over a 60-hr period. Control and experimental animals were sacrificed 72 hr after the initial injection, and the uteri were dissected out, blotted dry, and weighed. Two doses of commercial hCG (Profasi; Serono Laboratories) were also assayed as positive controls.

RESULTS

As shown in Fig. 1, we have replaced both the VP2 and VP1 coding regions of SV40 with the cDNAs of the complete α and β hCG preproteins. Most of the noncoding sequences at the 5′ end of the cDNAs were removed in order to place these inserts as closely as possible in the positions of the VP2 and VP1 genes of SV40.

The start codons (ATG) of VP2 and VP1 of SV40 are at residues 480 and 1417 of the SV40 genome (20), respectively. We have changed the sequences immediately adjacent to these residues in such a way as to insert the α and β hCG cDNAs without disrupting the late splicing signals used by the virus to generate its late transcripts. The *Ava* II site at residue 475 was filled in with Klenow polymerase and converted to a *Hin*dIII site by synthetic linkers. This construction conserves the late 19S acceptor splicing signal (21). The *Hin*dIII site at residue 1411 was similarly filled in and changed to an *Eco*RI site without disturbing the 16S acceptor splicing signal (22). In the final construction, the sequences T-T-C-A-G-G-T-C-C-A-T-G and T-C-T-A-A-A-A-G-C-T-T-A-T-G at the start positions of the VP2 and VP1 genes were replaced by T-T-C-A-G-G-T-C-C-A-A-G-C-T-T-G-C-A-T-G and T-C-T-A-A-A-A-G-C-T-G-G-A-A-T-T-C-C-G-G-A-T-G for the α and β hCG cDNAs, respectively. Thus, the α hCG insert has eight more nucleotides upstream from its ATG than the VP2 gene, and the β hCG insert has nine more than the VP1 gene. The overall length of αβSVVPI (4885 bp) was less than that of SV40 DNA (5243 bp) and very suitable for the formation of infective virus.

RNA produced by CV-1 cells infected with αβSVVPI was examined by formaldehyde/agarose gel electrophoresis followed by RNA blot transfer and hybridization to both α and β hCG cDNAs. As shown in Fig. 2 one large [2.1 kilobases (kb)] and one small (1.1 kb) poly(A)$^+$ RNA species were identified. The α hCG cDNA probe hybridized only to the larger species, whereas the β hCG cDNA probe hybridized to both the large and small RNAs. This result is expected in view of the published analysis of the variety of splices made during the processing of the late SV40 mRNAs (23) and indicates that the 2.1-kb mRNA is transcribed and processed as if it were the SV40 19S RNA species and that the 1.1-kb mRNA is transcribed and processed like the SV40 16S species. It should be noted that the 2.1-kb RNA of αβSVVPI contains both α and β hCG coding sequences; it thus hybridizes to both cDNA probes. The data indicate that the expression vector functions as it should with respect to transcription and mRNA processing. Fig. 3 depicts the generation of these RNAs from the recombinant late region of αβSVVPI. The RNA analysis also showed that the 1.1-kb RNA is produced in greater quantities than the 2.1-kb RNA. From the intensity of the RNA bands, we estimate that the

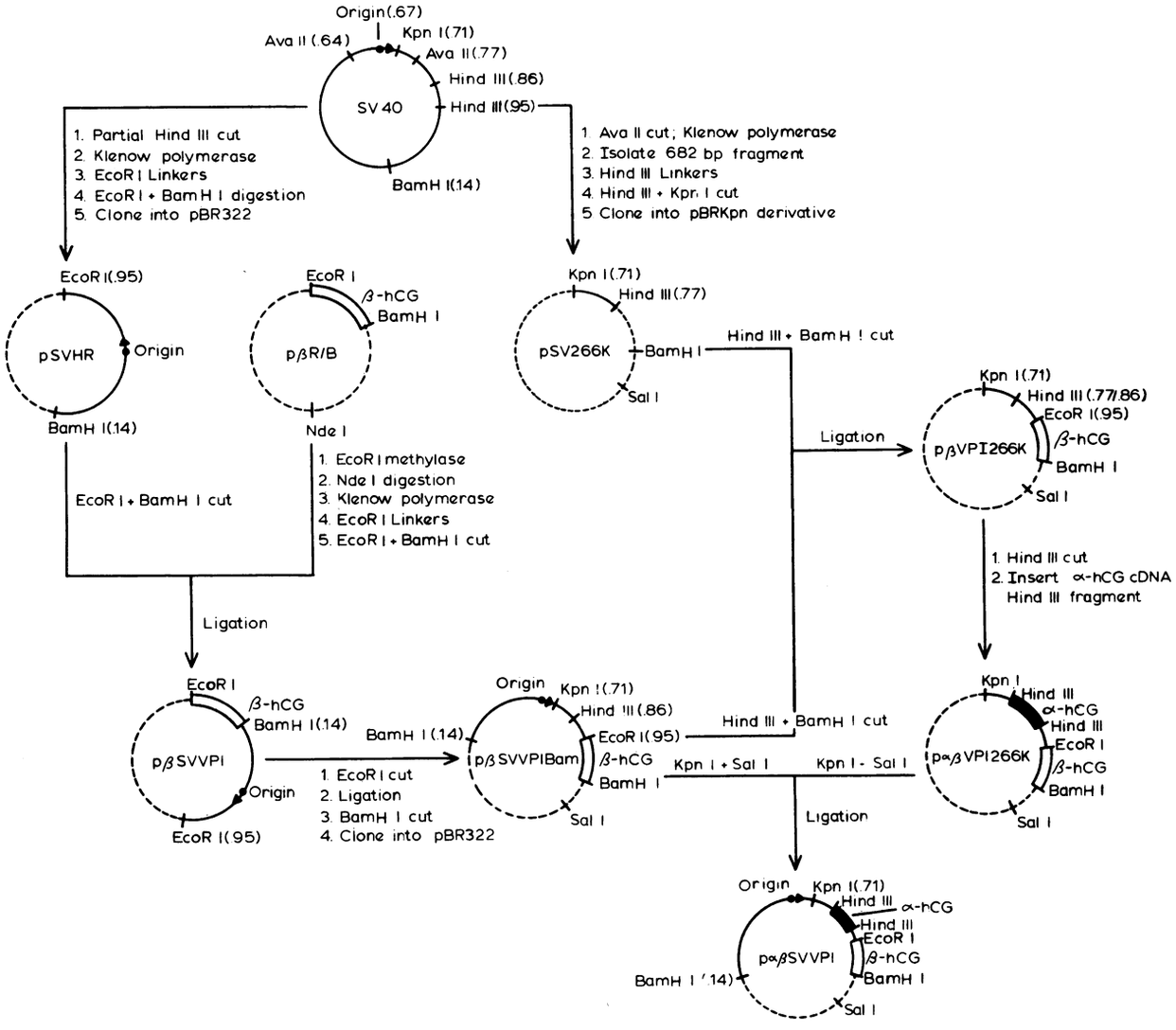

FIG. 1. Construction of $\alpha\beta$SVVPI. Restriction sites used in the construction are shown. The numbers in parentheses refer to map positions on the wild-type SV40 genome and are given in m.u. —, SV40 DNA; - - - -, PBR322 DNA; ■, α hCG cDNA; □, β hCG cDNA; ▶, SV40 late promoter.

ratio of 1.1-kb to 2.1-kb mRNA is ≈10. This is also the case in wild-type SV40 (23), where the 16S transcript is more abundant than the 19S RNA.

hCG was identified in the supernatants of CV-1 cells infected with $\alpha\beta$SVVPI by the immunoassays described. The presence of hCG in the medium suggests that it was processed correctly in CV-1 cells. However, because of the lytic nature of the system, further work is required to clearly establish this point. As shown in Fig. 4, hCG produced by CV-1 cells (rhCG) gave immunoassay results parallel to those of a reference preparation. We estimated that hCG dimer was produced at 1.1 mg/liter per 24 hr or 10^7 molecules of hCG per cell.

Fig. 5 presents evidence that dimeric hCG is produced by CV-1 cells infected with $\alpha\beta$SVVPI. Samples were run on Sephadex G-100 and assayed as described in the figure for individual subunits, dimeric hCG, and receptor binding activity. The results in Fig. 5*A* show that the receptor binding activity coelutes with hCG dimer (arrow), but does not coincide with the hCG β peak. The results given in Fig. 5*B* demonstrate that the α subunit is associated with dimeric material. Thus, dimer was shown to be present by *in vitro* receptor binding activity and by immunological means. The presence of free β subunit, constituting the bulk of the

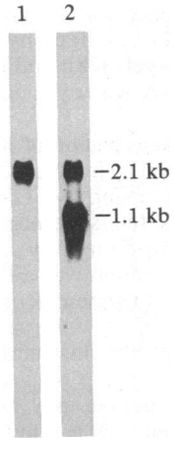

FIG. 2. RNA blot transfer of poly(A)$^+$ mRNA from CV-1 cells infected with $\alpha\beta$SVVPI. RNA transferred to nitrocellulose from a formaldehyde/agarose gel was immobilized and hybridized to ^{32}P-labeled α hCG cDNA (lane 1) or β hCG cDNA (lane 2). Autoradiography was performed after hybridization and washing. Size determinations are based on the use of denatured DNA markers (not shown).

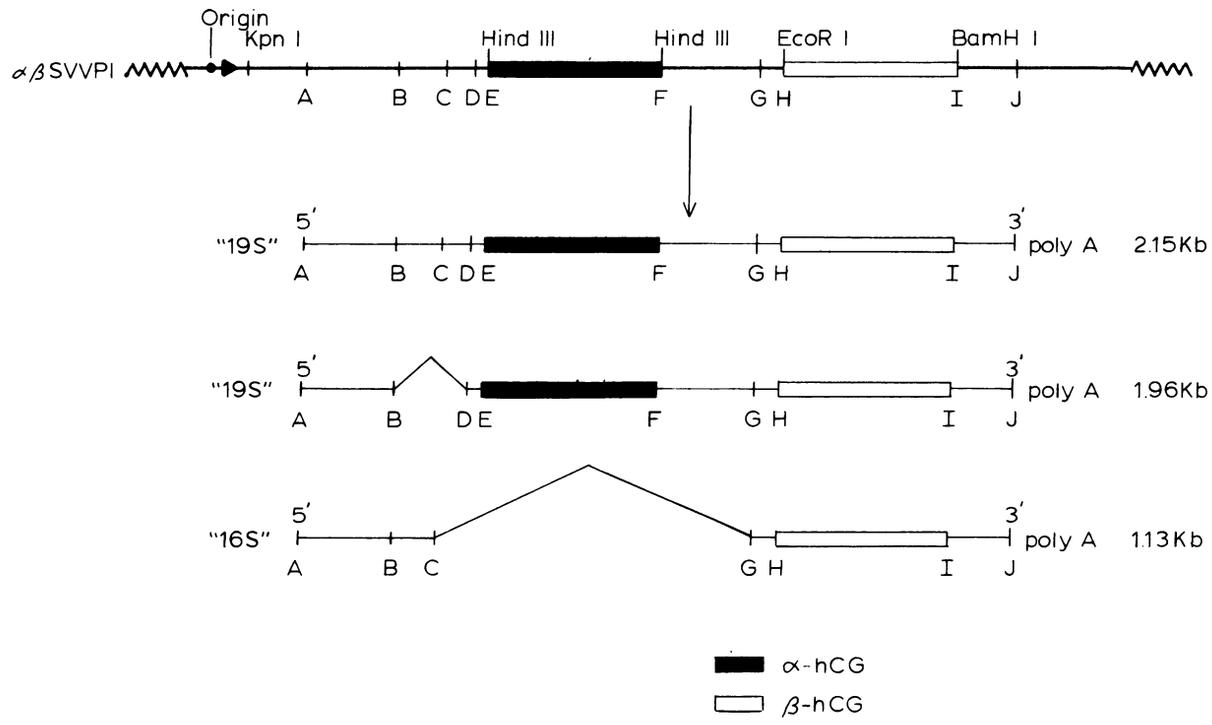

FIG. 3. Schematic representation of the biogenesis of late mRNAs from αβSVVPI. Relevant positions on the SV40 genome (21–23) are as follows: A, transcriptional initiation point at residue 243; B, 5′ end of major 19S splice junction at residue 291; C, 5′ end of major 16S splice at residue 444; D, 3′ end junction of the 19S splice at residue 476; E, *Ava* II site converted to a *Hin*dIII site as described in the text; F, *Hin*dIII site at position 968; G, 3′ end junction of the 16S splice at position 1381; H, former *Hin*dIII site at position 1411 converted to an *Eco*RI site; I, *Bam*HI site at position 2455; and J, 3′ termini of the late mRNAs at position 2592. RNA lengths were estimated on the assumption that there would be ≈200 adenylic acids at the 3′ ends. The SV40 late promoter is indicated by ▶.

immune-reactive material in Fig. 5*A*, is also indicated. Results of immune precipitation experiments (L. Anderson, personal communication) corroborate this conclusion in that subunits identical to those produced by choriocarcinoma cells were seen with a clear excess of hCG β subunit.

The biological activity of the rhCG dimer produced in CV-1 cells was determined *in vivo* by the mouse uterine weight assay (19). The increase in weight and size of the uteri with respect to those of the untreated female mice is a measure of biologically active hCG. As shown in Table 1, both crude tissue culture supernatants and pooled fractions from the Sephadex column in Fig. 5 were shown to possess activity in this assay. The results clearly demonstrate the ability of this system to produce biologically active hCG from cloned cDNAs.

DISCUSSION

We have shown that the SV40 late region is suitable for placing two distinct cDNAs under the control of the strong late promoter. The vectors that we have constructed may permit the expression, in one cell, of other multisubunit proteins such as LH, FSH, and immunoglobulins. In earlier experiments (data not shown), we were able to produce hCG by simply coinfecting CV-1 cells with a mixture of viruses containing either an α or β cDNA as single VP1 replacements. Thus, it is likely that this system can be used to produce many polypeptides in one cell by use of mixed infections with viruses containing two distinct inserts each. In addition to their basic importance in allowing the production of complex, multisubunit proteins, schemes such as the one presented in this report may provide the only efficient way to produce certain proteins. In this respect, it is interesting to note that the yields of β subunit from cells infected with single replacement virus are very low. They can be increased by replacing both VP2 and VP1 with the cDNA (unpublished results), but are still more than 1 order of magnitude less than those obtained when both the α and β cDNAs are expressed in the same cell. The molecular basis for this intriguing phenomenon remains to be explored.

Analysis of the mRNA produced by αβSVVPI showed 2.1-kb and 1.1-kb mRNAs, of which the latter hybridized only to β hCG cDNA. This result demonstrated that the 1.1-kb mRNA arose by use of the SV40 16S splice. We cannot

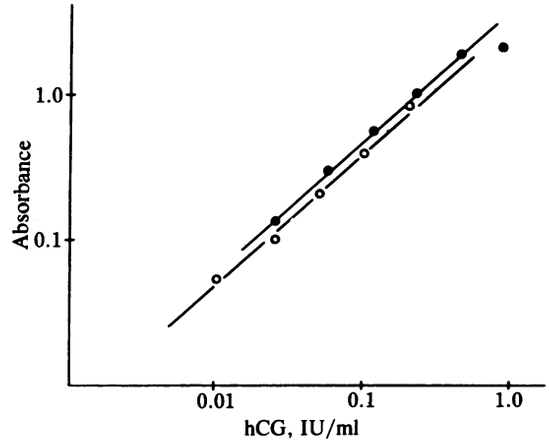

FIG. 4. Comparison of the immune reactivity of rhCG to that of urinary hCG. Enzyme-linked immunoassay was carried out on serial dilutions of rhCG and hCG purified from pregnancy urine (Serono Laboratories) using the Hybritech kit. The ordinate gives the absorbancy at 405 mn resulting from the alkaline phosphatase colorimetric assay used to quantitate the results. IU, international units.

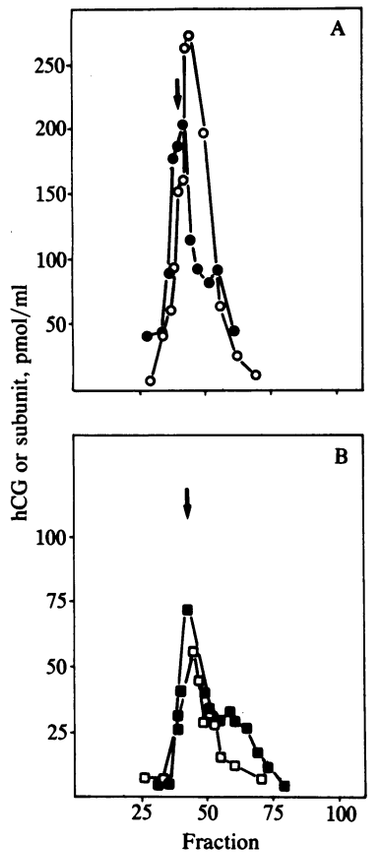

FIG. 5. Gel permeation chromatography of rhCG produced in CV-1 cells. Tissue culture supernatant was applied to a column (1.2 × 95 cm) of Sephadex G-100. Aliquots of each fraction were assayed for receptor binding (●) and β subunit (○) and for α subunit (■) and hCG dimer (□). The position of elution of a reference preparation of hCG (dimer) is indicated by the arrow.

yet state that the 2.1-kb mRNA, which hybridizes to both the α and β cDNAs, has been processed as if it were the 19S viral RNA because the removal of the short intron would not give a mRNA that can be resolved from an unspliced transcript. Similarly, it is not yet clear whether the SV40 polyadenylylation signal is used in αβSVVPI because the β hCG cDNA includes its own such signal, which overlaps with the translational termination codon (14) and is only 80 bp from the SV40 signal. An exact mapping of the mRNA termini will be required to settle this point. An interesting quantitative feature of the double replacement vector described here derives from the fact that the VP1 transcript and its product are more abundant than those of VP2 (23). This allows the manipulation of the relative amounts of the polypeptide chains to be produced by this system, which could be an important parameter for the production of some proteins.

The fact that a molecule as complex as hCG can be produced relatively simply and quickly by a viral system, in biologically active form, should soon provide solutions to many of the outstanding problems concerning the assembly of the glycoprotein hormones and assess which structural features are required for their activity. Genetically engineered changes in the structure of these molecules will be especially useful in this regard.

Table 1. Mouse uterine weight assays of rhCG

Sample	*n*	Uterine weight ± SEM, mg
Control	3	9.9 ± 2.5
Profasi (0.2 IU)	3	13.8 ± 3.7
Profasi (0.4 IU)	4	28.1 ± 4.2
rhCG (1:1)	3	15.4 ± 2.4
rhCG undiluted	3	30.1 ± 3.9
Pooled Sephadex (1:5)	3	17.3 ± 2.2

C57BL/6J females, 19 to 21 days old, were injected with commercially obtained hCG (Profasi; Serono Laboratories), dilutions of tissue culture supernatants, or a dilution of the pooled fractions 43–46 from the Sephadex column shown in Fig. 5. In each case, a total of 2 ml was used for inoculating each animal. Weights of freshly dissected blotted uteri were determined on an analytical balance. IU, international units.

We thank Ms. Kathy Schmidt for providing technical assistance in RIAs and Dr. Russell Pelham for his support throughout this work. This work was supported by a research contract from Serono Laboratories, Inc.

1. Canfield, R. E., Birken, S., Morse, J. H. & Morgan, F. J. (1976) in *Peptide Hormones*, ed. Parsons, J. A. (Univ. Park Press, Baltimore), pp. 299–315.
2. Pierce, J. G. & Parsons, T. F. A. (1981) *Annu. Rev. Biochem.* **50**, 465–495.
3. Kessler, M., Reddy, M. S., Shah, R. H. & Bahl, O. P. (1979) *J. Biol. Chem.* **254**, 7901–7908.
4. Kessler, M. J., Mise, T., Ghai, R. D. & Bahl, O. P. (1979) *J. Biol. Chem.* **254**, 7909–7914.
5. Birken, S. & Canfield, R. E. (1978) in *Structure and Function of the Gonadotropins*, ed. McKerns, K. W. (Plenum, New York), pp. 55–56.
6. Hamer, D. H. & Leder, P. (1979) *Nature (London)* **281**, 35–40.
7. Gruss, P., Efstratiadis, A., Karathanasis, S., Konig, M. & Khoury, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6091–6095.
8. Gething, M.-J. & Sambrook, J. (1981) *Nature (London)* **293**, 620–625.
9. Gheysen, D. & Fiers, W. (1982) *J. Mol. Appl. Genet.* **1**, 385–394.
10. Liu, C.-C., Yansura, D. & Levinson, A. D. (1982) *DNA* **1**, 213–221.
11. Gluzman, Y. (1982) *Eukaryotic Vectors* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
12. Gluzman, Y. (1982) *Cell* **23**, 175–182.
13. Fiddes, J. C. & Goodman, H. M. (1979) *Nature (London)* **281**, 351–356.
14. Fiddes, J. C. & Goodman, H. M. (1980) *Nature (London)* **286**, 684–687.
15. Kimura, G. & Dulbecco, R. (1972) *Virology* **49**, 79–81.
16. Sompayrac, L. M. & Danna, K. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7575–7578.
17. Penman, S. (1966) *J. Mol. Biol.* **17**, 117–130.
18. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
19. Loraine, J. A. & Brown, J. B. (1959) *J. Endocrinol.* **18**, 77–84.
20. Reddy, V. B., Thimmappeiya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L. & Weissman, S. (1978) *Science* **200**, 494–502.
21. Ghosh, P. K., Reddy, V. B., Swinscoe, J., Lebowitz, P. & Weissman, S. M. (1978) *J. Mol. Biol.* **126**, 813–846.
22. Bhaskara Reddy, V., Ghosh, P. K., Lebowitz, P. & Weissman, S. M. (1978) *Nucleic Acids Res.* **5**, 4195–4213.
23. Lebowitz, P. & Weissman, S. M. (1979) *Curr. Top. Microbiol. Immunol.* **87**, 43–172.