Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 6701–6705, November 1984
Biochemistry

Synthesis and glycosylation of the common α subunit of human glycoprotein hormones in mouse cells

(human chorionic gonadotropin cDNA/bovine papilloma virus vector/metallothionein gene/intracellular processing/secretion)

T. V. RAMABHADRAN, B. A. REITZ, AND D. C. TIEMEIER

Monsanto Co., 800 North Lindbergh Boulevard, St. Louis, MO 63167

Communicated by Howard A. Schneiderman, July 25, 1984

ABSTRACT **The synthesis and the post-translational modification of the α subunit of human glycoprotein hormones have been studied in a mouse cell. A full-length cDNA coding for the human α subunit has been expressed in mouse C127 cells under the control of mouse metallothionein regulatory sequences, using a bovine papilloma virus vector. Stable clones secreting the α subunit into the medium have been obtained. Two intracellular forms of 22,000 Da and 21,000 Da have been detected. Pulse–chase experiments suggest that the 22,000-Da form is exported, while the 21,000-Da form appears to remain intracellular. The secreted form of the α subunit migrates as a broad peak between 22,000 and 30,000 Da, suggesting further modification of the intracellular form prior to secretion. Both the secreted and the intracellular forms incorporate glucosamine label, indicating that at least a portion of the modification observed here is in the form of glycosylation.**

Thyrotropin, lutropin, follitropin, and chorionic gonadotropin are closely related glycoprotein hormones produced in the pituitary and the placenta. These hormones are composed of two non-covalently associated subunits: a common α subunit and a hormone-specific β subunit (1). Both subunits contain carbohydrate residues (1), which appear to be essential for the biological activity of the hormones (2–6). Because of these properties, the glycoprotein hormones provide an ideal model system for studying the synthesis of complex post-translationally modified proteins in homologous and heterologous mammalian cells.

The human genome contains a single gene encoding the common α subunit (7), which is expressed in the different cell types producing the glycoprotein hormones. In addition, a number of transformed cell lines produce the α subunit ectopically (8). In many cell types producing α and β subunits, two forms of α subunit, combined and free, are secreted, and these appeared to be modified differently (8–14). In this report, we describe the synthesis and processing of the α subunit in genetically engineered mouse cells.

We have introduced a full-length cDNA for the α subunit obtained from placental mRNA, synonymously referred to here as human chorionic gonadotropin-α (hCG-α), under the control of the regulatory elements of the mouse metallothionein (MT) I gene into C127 mouse cells using a bovine papilloma virus (BPV) as the vector (15). Isolation of stable clones secreting the human α subunit and the characterization of the protein produced by one such clone are described.

MATERIALS AND METHODS

Purified hCG-α subunit and rabbit anti-hCG-α antiserum used in the RIA were obtained from Accurate Chemicals (Westbury, NY). ^{125}I-labeled hCG and [6-^{3}H]glucosamine were obtained from New England Nuclear. The plasmid pCG-α and rabbit antisera to hCG-α were generous gifts of Irving Boime (Washington University School of Medicine, St. Louis, MO). The antisera used here were raised against native hCG-α, and reduced carboxymethylated hCG-α.

Full-length cDNA coding for the hCG-α subunit was isolated from pCG-α, a plasmid with the G·C-tailed cDNA insert at the *Pst* I site of pBR322 (7). The cDNA was isolated from pCG-α by restricting at the *Bam*HI site at the 5′ terminus (7, 16) and an *Hae* III site in pBR322 at position 3490 (17), which lies 3′ to the poly(A) addition signal and the 3′ G·C tail. The *Bam*HI/*Hae* III cDNA fragment was cloned into the M13 phage mp8 digested with *Bam*HI and *Hin*dII (18). The poly(A) addition signal and the G·C tail were eliminated by replacing the segment between the *Pst* I site in the coding region of the cDNA 7 2/3 codons upstream from the termination codon (16) and the *Hin*dIII site of M13 mp8 (18) with a chemically synthesized DNA fragment, so as to reconstruct the original coding sequence, including the terminator. The termination codon is followed immediately by sites for the enzymes *Xho* I and *Bgl* II (see Fig. 1). The accuracy of this reconstruction was verified by dideoxy sequencing (19). The resulting *Bam*HI/*Bgl* II fragment containing the 5′ untranslated sequences and the coding sequence of hCG-α was used in the construction of vectors shown in Fig. 1. Plasmids pJYMMT(E) and pBPV2308 used in these constructs were kindly provided by D. Hamer (National Institutes of Health).

Growth of C127 mouse cells, the DNA transfection procedure, and the isolation of BPV-transformed cell clones were carried out as described by Howley *et al.* (15). Transformed foci were subcloned into single wells of 24-well plates and the conditioned medium was assayed for hCG-α protein using RIA. Since the antibody used in the RIA recognized hCG-α subunit as well as intact hCG, we used ^{125}I-labeled hCG as the tracer and pure α subunit as the competing ligand. Antigen–antibody complexes were separated using Sepharose-linked goat anti-rabbit antibody (Miles).

Radioisotopic labeling experiments were performed in 9-cm^2 dishes. Cells were labeled with [^{35}S]methionine at 50–100 μCi/ml (1400 Ci/mmol; 1 Ci = 37 GBq; Amersham) in methionine-free modified Eagle's medium/2% dialyzed fetal bovine serum. [^{3}H]Glucosamine (43 Ci/mmol) labeling was done in glucose-free Dulbecco's modified Eagle's medium (8)/2% dialyzed fetal bovine serum, at a final concentration of 100 μCi/ml. After labeling, cells were lysed in buffer A (phosphate-buffered saline/1% Nonidet P-40/0.5% deoxycholate) containing 1000 kallikrein inhibitory units (KIU) of the protease inhibitor Trasylol (Sigma). The lysate was spun in an Eppendorf centrifuge to remove insoluble cell debris and was stored at −20°C. For secretion studies, pulse-la-

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.

Abbreviations: BPV, bovine papilloma virus; MT, metallothionein; hCG, human chorionic gonadotropin.

beled cells were incubated for 3 hr in complete medium. After the chase, the medium was spun to remove detached cells and was stored at −20°C after the addition of 1000 KIU of Trasylol.

Labeled cell lysates, and chase media brought to 1× in buffer A were immunoprecipitated with native α-specific rabbit antiserum. Antigen–antibody complexes were isolated by binding to formaldehyde-fixed *Staphylococcus aureus* (Miles), reduced with 2-mercaptoethanol, and analyzed on $NaDodSO_4$ 15% polyacrylamide gels as described by Laemmli (20).

RESULTS

Vector Construction. Construction of the BPV vector pMON1040 used in the transfection of C127 cells is shown in Fig. 1. The *Bam*HI/*Bgl* II cDNA fragment containing the 5′ flanking sequences and the coding sequence of hCG-α was introduced into the unique *Bgl* II site of the plasmid pJYMMT(E) (21) to obtain pMON1038. The *Bgl* II site in pJYMMT(E) lies within the mouse MT-I genomic fragment, 64 bases downstream from the cap site of the MT transcript, just preceding the ATG initiation codon (22). In this configuration, transcription from the MT-I promoter in pMON1038 would result in a chimeric mRNA with the 5′ untranslated leader of the MT-I mRNA followed, respectively, by the hCG coding sequence and the coding sequence of the *MT* gene. The downstream *MT* gene provides the introns and the poly(A) addition signal putatively required for the proper processing of the mRNA (see ref. 23). In addition to the *MT* gene, pJYMMT(E) also contains the entire simian virus 40 genome linked to a "poison minus" pBR322 derivative and is, therefore, capable of replicating in monkey cells (21). DEAE dextran-mediated transfection of pMON1038 into CV-1 monkey cells resulted in the production of hCG-α (not shown).

To construct the BPV vector pMON1040, the *Bam*HI/*Sal* I fragment of pMON1038, including the hCG-MTI chimeric gene and the poison minus pBR322 sequences, was ligated to a full-length BPV genome with *Bam*HI and *Sal* I termini. Calcium phosphate-precipitated pMON1040 DNA was used to transfect mouse C127 cells as described by Howley *et al.* (15).

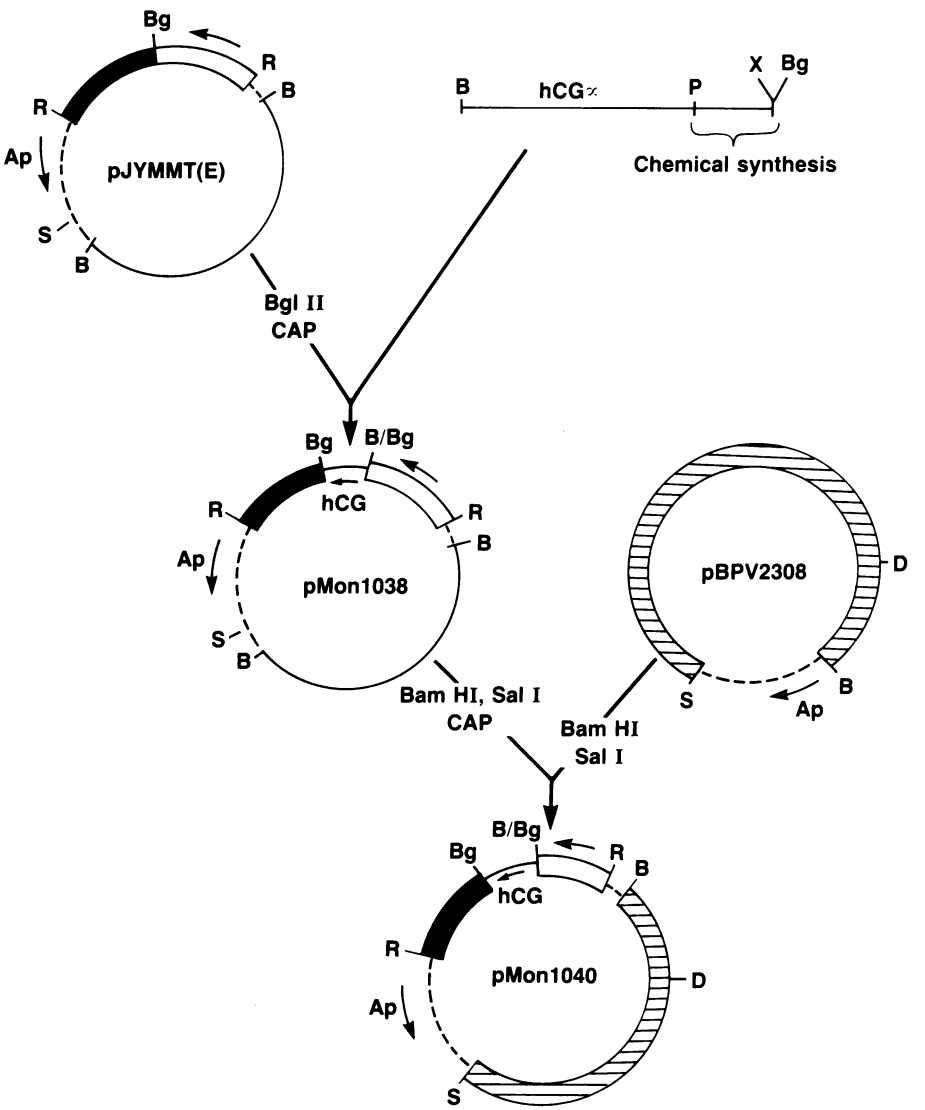

FIG. 1. Construction of pMON1040 BPV vector for the transfection of C127 cells. Open boxes represent MT-I promoter region; solid boxes, MT-I coding sequence and 3′ flanking sequences; cross-hatched boxes, BPV sequences; dotted lines, pBR322 sequences. Continuous line between the pair of *Bam*HI (B) sites in pJYMMT (E) and pMON1038 represents the simian virus 40 genome. The hCG-α sequences are also represented by a continuous line and are labeled. Enzymes: B, *Bam*HI; Bg, *Bgl* II; D, *Hin*dIII; P, *Pst* I; R, *Eco*RI; S, *Sal* I; X, *Xho* I; B/Bg, fusion joint of *Bam*HI and *Bgl* II sticky ends; CAP, calf liver alkaline phosphatase; Ap, ampicillin resistance gene.

Isolation and Characterization of hCG-α Producing Clones. Two to three weeks after the transfection, 72 transformed foci were cloned into individual wells of 24-well plates. After 1 week of growth, the amount of hCG-α secreted into the culture medium during a 48-hr period was measured by RIA. Production levels of hCG-α ranged from undetectable to 300 ng/ml in the clones tested. No hCG-α signals were detected in the medium of a BPV-transformed clone that did not contain hCG-related sequences on the vector. Five clones that appeared to produce the highest level of hCG-α were studied further. Table 1 shows the levels of hCG-α secreted by the five clones during a 24-hr period. Addition of noncytotoxic levels of Cd^{2+} or Zn^{2+} to the medium caused an increase in the levels of secreted hCG-α. This result is consistent with the idea that transcription of the hCG-α-producing transcript is under the control of the MT promoter, which has been shown to be inducible by heavy metals (21, 24). However, the extent of response to the metal ions shows considerable variability, with induction ratios varying between 2- and 5-fold. A similar decrease in the responsiveness of the MT-I promoter to metal ions has been observed in BPV-derived transformants producing human growth hormone (25).

In general, when clones were tested by RIA at 48 hr, >95% of hCG-α was found in the medium, indicating proficient secretion and the absence of high levels accumulating intracellularly.

In all further studies, described below, we used the clone 20 described in Table 1. Fig. 2 shows the kinetics of hCG-α secretion by this line. The hCG-α protein is detectable over background as early as 3 hr, but the increase is small compared to that which becomes detectable at 9 hr. Pulse–chase experiments described below (see Fig. 4) confirm the synthesis and secretion during these early periods. This line secretes ≈300 ng of hCG-α per 10^6 cells per day and has been stable over the past 4 months. We have detected BPV sequences in the Hirt supernatants (26) of clone 20 by Southern gel analysis (27), indicating episomal replication of the BPV vector in the cells (28).

Characterization of the Intracellular and Secreted Forms of hCG-α. The α subunit of glycoprotein hormones is synthesized eutopically in the pituitary and placenta, and ectopically in a number of tumor cell types (8–14). The intracellular and secreted forms of this protein have been extensively characterized in the studies cited. We examined the size and the extent of glycosylation of the α subunit synthesized in clone 20 in the following experiments.

Cells were pulsed for 1 hr with either [^{35}S]methionine or [6-^{3}H]glucosamine. Cell lysates were prepared immediately after the pulse or after a 3-hr chase in nonradioactive medium. Immunoprecipitates of the lysates and the chase medium using antiserum against native α subunit were analyzed on $NaDodSO_4$/polyacrylamide gels. Fig. 3 shows the data

Table 1. Response of hCG-α secretion by pMON1040-derived clones to Cd^{2+} and Zn^{2+}

Clone	hCG-α, ng/ml			
	No addition	Cd^{2+} (1 μM)	Cd^{2+} (5 μM)	Zn^{2+} (50 μM)
1	50	66	100	95
5	270	500	560	280
7	93	300	500	495
18	180	190	320	540
20	200	250	470	280

Cells (10^6) from each clone grown overnight in 9-cm^2 dishes were exposed to medium containing Cd^{2+} or Zn^{2+} ions for 24 hr. hCG-α in the medium was assayed by RIA. Background hCG-α-reactive material in medium of a similarly treated transformed cell line derived using an unrelated BPV vector was <2 ng/ml.

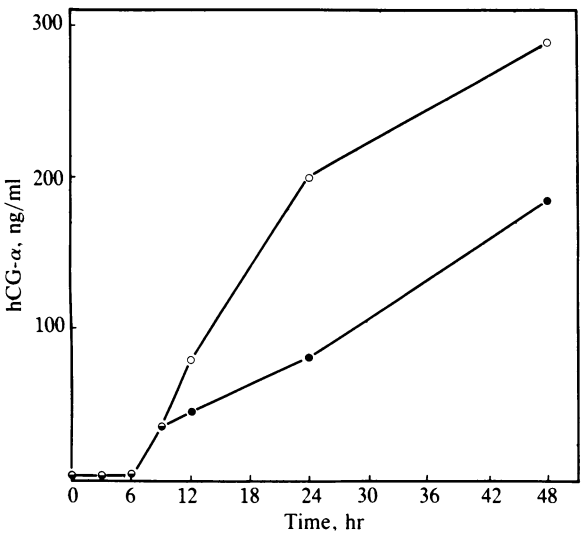

FIG. 2. Secretion of hCG-α by pMON1040 clone 20 into culture medium. Cells grown in 9-cm^2 dishes were exposed to fresh medium with or without 5 μM Cd^{2+} at 0 hr. Aliquots of medium at various times shown were assayed for hCG-α by RIA. Closed circles, medium alone; open circles, medium containing 5 μM Cd^{2+}.

for [^{35}S]methionine labeled lysates and media. Two bands of ≈22 and 21 kDa are seen in cell lysates immediately after pulse (lanes 4 and 5). Neither of these bands is seen when immunoprecipitations are carried out in the presence of excess unlabeled hCG-α (not shown). The 22-kDa band comigrates with the ^{125}I-labeled hCG-α standard (lane 1) obtained by dissociation of ^{125}I-labeled native hCG (see *Materials and Methods*), and it disappears during the subsequent chase (lane 6). The 21-kDa form, however, persists in the intracellular compartment throughout the chase period. These data suggest that the 22-kDa form may represent the secreted component of hCG-α, whereas the 21-kDa form may remain intracellular and be degraded, or it may be exported slowly.

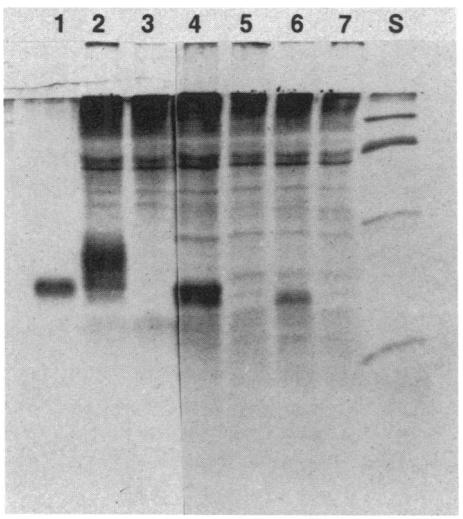

FIG. 3. Intracellular and secreted forms of hCG-α in pMON1040 clone 20. Cells in 9-cm^2 dishes were pulsed with [^{35}S]methionine for 1 hr and either lysed immediately or chased for 3 hr in complete medium and then lysed. Aliquots of lysates and medium were immunoprecipitated with rabbit antiserum against native hCG-α or with a nonspecific rabbit serum. Lanes 4 and 5, lysates after pulse; lanes 6 and 7, lysates after 3-hr chase; lanes 2 and 3, medium from chase. Lanes 2, 4, and 6, hCG-α antiserum; lanes 3, 5, and 7, nonspecific antiserum. Lane 1, ^{125}I-labeled hCG-α marker from dissociated native ^{125}I-labeled hCG. Lane S, radioactive markers (top to bottom) 94,000, 67,000, 30,000, and 12,000 Da.

Since the α subunit is synthesized ectopically in many tumor cells and since the C127 cells were derived from a mammary tumor (29), it was necessary to rule out the possibility that the observed bands were due to endogenous mouse α gene expression. We examined lysates from pulse-labeled cells transformed with a BPV hybrid carrying an insert unrelated to hCG-α. This lysate as well as clone 20-derived lysate were immunoprecipitated using two hCG-α-specific antisera raised against native and reduced carboxymethylated α subunit. Both antisera precipitated the 22- and 21-kDa bands from the lysates of clone 20. However, neither of the α-specific bands shown in Fig. 3 were immunoprecipitated from the lysate of the unrelated BPV transformant with either antisera, indicating that the two forms of hCG-α seen in the lysate of clone 20 are specific to the hCG-α gene in pMON1040.

Fig. 3 (lane 2) shows the immunoprecipitation of hCG-α-specific material secreted into the medium during the chase period. A disperse band of material running between 22 and 30 kDa is observed. The increase in size of the secreted subunit is in agreement with observations of increase in the molecular size of secreted free α subunit from the pituitary, placenta, or ectopically producing cells (8–14), and it presumably represents additional glycosylation of the intracellular form during secretion (14).

Fig. 4 shows results of a pulse–chase experiment using [^{3}H]glucosamine to label the sugar moieties attached to the proteins. In pulsed cell lysates (lane 4), two bands corresponding in molecular size to the 22- and 21-kDa forms shown in Fig. 3 are seen. The lower molecular size, 21-kDa form, incorporates more label than the 22-kDa form. During the 3-hr chase (lane 6), both α-specific bands show an increase in intensity. The differences in the kinetics of [^{35}S]methionine and [^{3}H]glucosamine label incorporations are likely to represent the differences in the flow of these precursors through their respective intracellular pools. Nontheless, these data show that both the intracellular forms of the α subunit contain sugar moieties attached to them. The secreted form of the protein also labels with glucosamine (lane 2) and shows the increase in molecular size similar to that seen in the ^{35}S-labeling experiment described in Fig. 3. However, in contrast to the ^{35}S-labeled lysates, small amounts of the higher molecular size form are observed in the lysates of glucosamine labeled cells. The data in Figs. 3 and 4 taken together show that the major species of α subunit-related material in the lysate and in the medium are glycosylated. The immunoprecipitates of ^{35}S-pulsed cell lysates using two α-specific antisera, against native and reduced carboxymethylated α subunit, revealed no bands other than the two forms described above. Thus, these forms appear to be the only predominant α subunit-related proteins in the intracellular space.

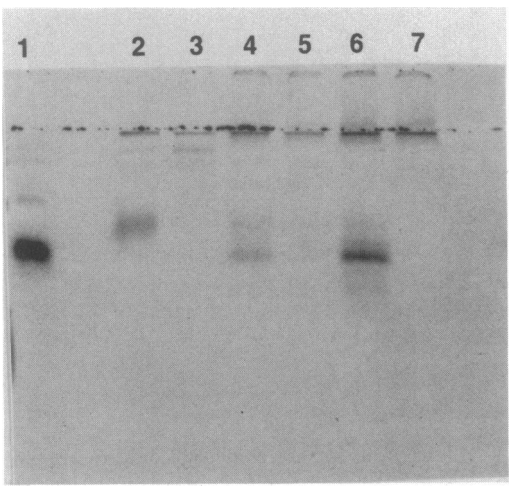

FIG. 4. Glycosylation of hCG-α produced by pMON1040 clone 20. [6-^{3}H]Glucosamine-labeled cell lysates and chase media (see Fig. 3 for experimental details) were immunoprecipitated with antibodies to native α subunit or with a nonspecific antibody. Lanes 2 and 3, chase medium; lanes 4 and 5, lysates after pulse; lanes 6 and 7, lysates from pulse-chased cells. Lane 1, ^{125}I-labeled hCG-α marker from dissociated native ^{125}I-labeled hCG. The light band in lane 1 migrating behind the dark hCG-α band corresponds to hCG-β.

DISCUSSION

We have expressed the full-length cDNA for the α subunit of human glycoprotein hormones under the control of the mouse MT-I promoter in mouse C127 cells using a BPV vector. Transformed clones synthesize and secrete glycosylated hCG-α-related polypeptide continuously. In a number of clones examined, >95% of the α subunit is found in the culture medium, indicating very efficient secretion. The highest producing clones secretes ≈300 ng of hCG-α per 10^6 cells per day.

Although the primary sequence and the locations of the glycosylation sites are highly conserved among the α subunits from various mammalian species (1, 16, 30–32), there is ≈25% amino acid sequence divergence between human and mouse α subunits (32). It was, therefore, of great interest to study the post-translational processing of the human α subunit in a heterologous mouse cell. We characterized the intracellular and the secreted forms of hCG-α in one of the high producing clones (clone 20), using pulse–chase experiments.

Two equally abundant forms of hCG-α-related proteins migrating at 22 kDa and at 21 kDa were observed in the [^{35}S]methionine-labeled lysates of clone 20. The sizes of the intracellular forms are considerably larger than the ≈10 kDa expected from the primary sequence of the protein (1). The lower electrophoretic mobility of the intracellular forms seen here is explained by the presence of carbohydrate on both the species. The 22-kDa intracellular form comigrates with the α subunit of the dissociated urinary hCG standard, and pulse–chase experiments indicate that it is efficiently secreted. On the other hand, based on similar pulse–chase experiments, the 21-kDa form is apparently persisting in the intracellular space. Moreover, hCG-α-reactive material does not accumulate intracellularly (RIA data), suggesting that continuous production of the 21-kDa form may be counterbalanced by slow degradation or release. Alternatively, the 21-kDa form may be converted slowly to the 22-kDa form or an as yet unidentified form and subsequently secreted.

Native hCG and the free α subunit are synthesized by the human placenta (12) and human choriocarcinomas (8). In both cases, two intracellular forms of 18 kDa and 15 kDa, bearing a precursor–product relationship, are observed. Our data show that the intracellular processing of the human α subunit in mouse cells follows a different pathway. Interestingly, an intracellular form corresponding in size to the α subunit of secreted native hormones has been observed in normal and tumor cells derived from rodent pituitaries (13, 33, 34). Thus the 22-kDa form seen in this C127 clone may be the product of a pathway specific to pituitary cells or to the rodent species.

The 21-kDa nonsecreted form we have observed has not been reported in any of the studies cited above. However, Ruddon *et al.* (8) have reported the presence of nonsecreted hCG-α in the intracellular compartment of a mammary carcinoma and a neuroblastoma line. Coincidentally, the C127 cells are derived from a mammary carcinoma (29), and therefore, the 21-kDa nonsecreted form may be analogous to the nonsecreted hCG-α reported by Ruddon *et al.* (8).

The observation that the α subunit secreted by clone 20

has a larger molecular size than the α subunit dissociated from hCG is extremely interesting. Free α subunit is secreted eutopically and ectopically by many cell types of human, bovine, and rodent origin. In all these cases, the secreted free form has been shown to have a larger molecular size than the α subunit secreted in combination with the corresponding β subunit (8–14, 35). The increase in molecular size is believed to represent further modification of the free α subunit prior to secretion. Apparently, the modifying enzymes in mouse cells may recognize the structural features of the human α subunit that signal this type of modification. The mouse cell form of hCG-α might, like the free α subunit purified from bovine pituitaries (14), carry an additional O-linked oligosaccharide normally absent in the combined α subunit. However, there are no data here to suggest that. In any event, this high molecular size α is seen in only small amounts in cell lysates of clone 20. This is true of all cases cited above as well and suggests that the additional modification of the secreted α occurs as a terminal step in secretion.

C127 cells and BPV-transformed C127 cells apparently do not produce large quantities of mouse α subunit ectopically. In control experiments using C127 cells transformed by a BPV vector unrelated to pMON1040 used here, we detected no secretion of α-like material. Labeled cell lysates from this line immunoprecipitated with two independent polyclonal antisera against hCG-α did not show any α-specific bands on gels. Although there may be production of mouse α subunit below the sensitivity of our assay, the simplest interpretation of these results is that BPV-transformed C127 cells do not produce mouse α ectopically. This would suggest that the mechanisms that result in the modification, and, more interestingly, hypermodification of the α subunit are not limited to cell types that produce the α subunit eutopically or ectopically.

We thank Drs. Irving Boime and Dean Hamer for the generous gift of plasmids, Debbie Connors and Dr. Steve Adams for oligonucleotide synthesis, and Patsy Guenther for excellent typing. Critical reading of the manuscript by Dr. Irving Boime and Chris Corless is greatly appreciated.

1. Pierce, J. G. & Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495.
2. Chem, H.-C., Shimohigashi, Y., Dufau, M. L. & Catt, K. J. (1982) *J. Biol. Chem.* **257,** 14446–14452.
3. Goverman, J. M., Parsons, T. F. & Pierce, J. G. (1982) *J. Biol. Chem.* **257,** 15059–15064.
4. Kalyan, N. K. & Bahl, O. P. (1983) *J. Biol. Chem.* **258,** 67–74.
5. Keutman, H. T., McIlroy, P. J., Berget, E. R. & Ryan, R. J. (1983) *Biochemistry* **22,** 3067–3072.
6. Sairam, M. R. & Manjunath, P. (1983) *J. Biol. Chem.* **258,** 445–449.
7. Boothby, M., Ruddon, R. W., Anderson, C., McWilliams, D. & Boime, I. (1981) *J. Biol. Chem.* **256,** 5121–5127.
8. Ruddon, R. W., Hanson, C. A., Bryn, A. H. & Anderson, C. (1980) in *Chorionic Gonadotropin*, ed. Segal, S. J. (Plenum, New York), pp. 295–315.
9. Benveniste, R., Conway, M. C., Puett, D. & Rabinowitz, D. (1979) *J. Clin. Endocrinol. Metab.* **48,** 85–91.
10. Dean, D. J., Weintraub, B. D. & Rosen, S. W. (1980) *Endocrinology* **106,** 849–858.
11. Chin, W. W., Habener, J. F., Martorana, M. A., Keutman, H. T., Kieffer, D. J. & Maloof, F. (1980) *Endocrinology* **107,** 1384–1392.
12. Ruddon, R. W., Hartle, R. J., Peters, B. P., Anderson, C., Huot, R. I. & Stromberg, K. (1981) *J. Biol. Chem.* **256,** 11389–11392.
13. Hoshina, H. & Boime, I (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7649–7653.
14. Parsons, T. F., Bloomfield, G. A. & Pierce, J. G. (1983) *J. Biol. Chem.* **258,** 240–244.
15. Howley, P. M., Sarver, N. & Law, M. F. (1983) *Methods Enzymol.* **101,** 387–403.
16. Fiddes, J. C. & Goodman, H. M. (1979) *Nature (London)* **281,** 351–356.
17. Sutcliffe, G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77–90.
18. Messing, J. & Viera, J. (1982) *Gene* **19,** 269–276.
19. Sanger, F., Nickelson, S. & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
21. Hamer, D. H. & Walling, M. (1982) *J. Mol. Appl. Genet.* **1,** 273–288.
22. Glanville, N., Durnam, D. M. & Palmiter, R. D. (1981) *Nature (London)* **292,** 267–269.
23. Rigby, P. W. J. (1982) in *Genetic Engineering*, ed. Williamson, R. (Academic, New York), Vol. 3, pp. 83–141.
24. Mayo, K. E. & Palmiter, R. D. (1981) *J. Biol. Chem.* **256,** 2621–2624.
25. Pavlakis, G. N. & Hamer, D. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 397–401.
26. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
27. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
28. Law, M. F., Lowy, D. R., Dvoretzky, I. & Howley, P. M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2727–2731.
29. Dvoretzky, I., Shober, R., Chattopadhyay, S. K. & Lowy, D. R. (1980) *Virology* **103,** 369–375.
30. Chin, W. W., Kronenberg, H. M., Dee, P. C., Maloof, F. & Habener, J. F. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5329–5333.
31. Godine, J. E., Chin, W. W. & Habener, J. F. (1982) *J. Biol. Chem.* **257,** 8368–8371.
32. Nilson, J. H., Thomason, A. R., Cserbak, M. T., Moncman, C. L. & Woychik, R. P. (1983) *J. Biol. Chem.* **258,** 4679–4682.
33. Chin, W. W., Habener, J. F., Kieffer, D. J. & Maloof, F. (1978) *J. Biol. Chem.* **253,** 7985–7988.
34. Magner, J. A. & Weintraub, B. D. (1982) *J. Biol. Chem.* **257,** 6709–6715.
35. Ridgway, C. E., Keiffer, D. J., Ross, D. S., Downing, M., Mover, H. & Chin, W. W. (1983) *Endocrinology* **113,** 1587–1591.