Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 463-473

DETECTION OF TWO PRECURSORS TO EACH OF THE SUBUNITS OF HUMAN CHORIONIC GONADOTROPIN TRANSLATED FROM PLACENTAL mRNA IN THE WHEAT GERM CELL-FREE SYSTEM

John E. Godine, William W. Chin and Joel F. Habener

Laboratory of Molecular Endocrinology and Endocrine Unit, Massachusetts General Hospital, Howard Hughes Medical Institute Laboratories, Harvard Medical School, Boston, MA 02114

Received November 18, 1981

SUMMARY: Polyadenylated RNA prepared from first trimester human placenta was translated in a membrane-free cell-free system derived from wheat germ. Analysis of the [^{35}S]methionine-labeled products by SDS-polyacrylamide electrophoresis demonstrated two proteins with apparent M_rs of 14,500 and 16,000 that were specifically immunoprecipitated by antiserum to reduced and carboxylated bovine LH_α, and two different proteins with apparent M_rs of 18,500 and 21,000 that were specifically immunoprecipitated by antiserum to hCG_β. None of these products was sensitive to cleavage by endoglycosidase H, whereas the M_r 21,000 product precipitated by antisera to bovine LH_α and to hCG_α from translations supplemented by canine pancreatic microsomes was processed to a product with M_r 13,000 by endoglycosidase H. We suggest that the two forms of the α and β subunit precursors could arise from the translation of two distinct mRNAs encoding each subunit.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a placental hormone which supports the function of the corpus luteum during early pregnancy. Production of the hormone increases to a peak at the end of the first trimester and then declines gradually during the remainder of gestation.

As with the pituitary hormones thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), hCG is composed of two distinct non-covalently associated glycoprotein subunits, designated α and β (1-3). Within a species, the α subunits of these hormones are virtually identical, whereas, the β subunits, despite considerable homology, differ sufficiently to confer upon each hormone its own specific biologic properties. In particular, hCG_β contains a COOH-terminal extension of approximately 30 amino acids that distinguish it from the other glycoprotein hormones. Each subunit of hCG has two branched carbo-

0006-291X/82/020463-11$01.00/0

hydrate side chains in N-glycosidic linkage to asparagine residues. In addition, hCG_β has four oligosaccharide chains linked to serine residues in the COOH-terminal portion of the protein (1-3).

In recent years, considerable progress has been made in elucidating the details of the processes involved in the biosynthesis of hCG and the pituitary glycoprotein hormones. Messenger RNA extracted from first trimester human placenta has been translated in a variety of cell-free systems. Immunoprecipitation techniques have resulted in the identification of precursors to the α and β subunits as products with apparent M_rs of 14,000 and 18,000, respectively, on SDS-polyacrylamide gels (4,5). Fractionation of the messenger RNA has demonstrated that these precursors are translated from separate mRNAs, and hence are presumably encoded by separate genes (5). When the cell-free translations are performed in the presence of microsomal membranes, the 14,000 dalton hCG_α precursor is processed to a 21,000 dalton glycosylated form (6,7). In addition, a variety of glycosylated precursors of both subunits have been demonstrated by pulse-chase labeling studies using cell lines which produce hCG or isolated subunits eutopically or ectopically (8-10). Furthermore, Fiddes and Goodman have used recombinant DNA technology to clone complementary DNA for each subunit of hCG (11-12). This procedure has provided the nucleotide sequence of the cDNA encoding the structural region and NH_2-terminal signal sequence of both subunits as well as parts of the 5' and 3' untranslated regions.

We have recently translated placental mRNA in cell-free systems in parallel with our studies of the pituitary glycoprotein hormones (13-14). For both the α and β subunits, we have identified by immunoprecipitation two separate precursor forms, the smaller of which, in each case, corresponds in apparent molecular weight with the precursors previously identified by other investigators. We suggest that each of the two α and β subunit precursors may be encoded by a separate mRNA.

MATERIALS AND METHODS

The isolation of polyadenylated RNA, translation of messenger RNA in the wheat germ extract cell-free system, and SDS-polyacrylamide gel electrophoresis of translation and immunoprecipitation products were performed as described previously (13,14).

Translations were performed in reaction mixtures containing 2 mCi/ml of [^{35}S]methionine (800 to 1,000 Ci/mmol, New England Nuclear, Boston, MA). SDS-polyacrylamide gels were treated with Enhance (New England Nuclear, Boston, MA) before drying. Autoradiograms were performed by exposing the dry gels to Kodak SB-5 film at -70°C for 3-10 days. Apparent molecular weights were determined by comparison with a series of molecular weight markers - lysozyme, 14,300; β-lactoglobulin, 18,000; trypsinogen, 24,000; ovalbulin, 45,000; bovine plasma albumin 66,000 (Sigma, St. Louis, MO).

Immunoprecipitations. Immunoprecipitations were performed using antiserum to reduced and carboxymethylated bovine LH_α, generously provided by Dr. J.G. Pierce (UCLA), and antiserum to hCG_α and hCG_β supplied by the Hormone Distribution Program of the National Pituitary Agency. Ten microliters of translation mixture was made 25 mM in Tris HCl, pH 7.4, 0.33% (w/v) in SDS and 0.33% (v/v) in 2-mercaptoethanol in a final volume of 15 µl and was heated to 100°C for two minutes. To this was added 85 µl of phosphate-buffered saline (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 1% (v/v) Triton X-100, 10^3 U/ml Trasylol and 2.5 mg/ml human serum albumin. The mixture was centrifuged for 15 minutes at 12,000 x g and the supernatant was incubated overnight at 4°C with 1 or 2 µl of the appropriate antiserum. In control reactions, the specific antiserum was replaced by nonimmune serum, or excess unlabeled subunit was added prior to the addition of specific antiserum. Following the overnight incubation, 25 µl of Staphylococcus Aureus cells (Pansorbin,Calbiochem-Behring Corp.) was added to each 100 µl reaction mixture. After a ten minute incubation at room temperature and centrifugation, the supernatant was discarded and the pellet was washed with cold phosphate-buffered saline as previously described (13). The pellet was then resuspended in a reducing and denaturing sample electrophoresis buffer (13) and was heated for one hour at 37°C followed by three minutes at 100°C. The tubes were centrifuged at 12,000 x g for one minute and the supernatant was applied to linear gradient SDS-polyacrylamide gels.

Membrane Processing. Microsomes from dog pancreas were prepared by the method of Katz el al. (15) by Dr. J. Majzoub. In experiments involving processing of translation products, 1.0 µl (115 A 280 units/ml) of these microsomal membranes was added directly to the translation mixtures just before the addition of the appropriate RNA.

Endo-β-acetylglucosaminidase H Digestion. The translation or immunoprecipitation products to be digested were mixed with or dissolved in buffer to obtain final concentrations of 150 mM Tris (pH 8.8), 1% (v/v) β-mercaptoethanol. The solution was heated to 100°C for two minutes. After the solution cooled, 0.6 volume of 0.3 M sodium citrate (pH 5.3) was added. Endo-β-acetyglucosaminidase H, kindly provided by Dr. P. Robbins (M.I.T.), was added to obtain a final concentration of 3 µg/ml and the mixtures were incubated at 37°C for 6 to 20 hours. Sample electrophoresis buffer was added and the mixtures were heated to 100°C for two minutes and then were subjected to electrophoresis on SDS-polyacrylamide gels.

RESULTS

Analysis of the [^{35}S]methionine-labeled translation products of first trimester placental mRNA showed a major band with apparent M_r= 25,000 as well as several minor bands (Figure 1A, lane 2). The major product represents a precursor to placental lactogen (16,17). To demonstrate structural relationships between specific cell-free translation products and the authentic subunits of hCG, immunoprecipitation reactions were performed using specific antisera. Immunoprecipitation using antiserum to reduced and carboxymethylated bovine LH_α (RCM bLH_α) identified two products of apparent M_rs = 14,500 and 16,000 (Figures 1a, lane 4, and 1b, lane 4). Immunoprecipitation of both bands was inhibited in the presence of excess unlabeled hCG_α (40 µg/ml) but not in the presence of excess unlabeled hCG_β (40 µg/ml) (Figure 1b, lanes 5 and 6, respectively). As has been noted

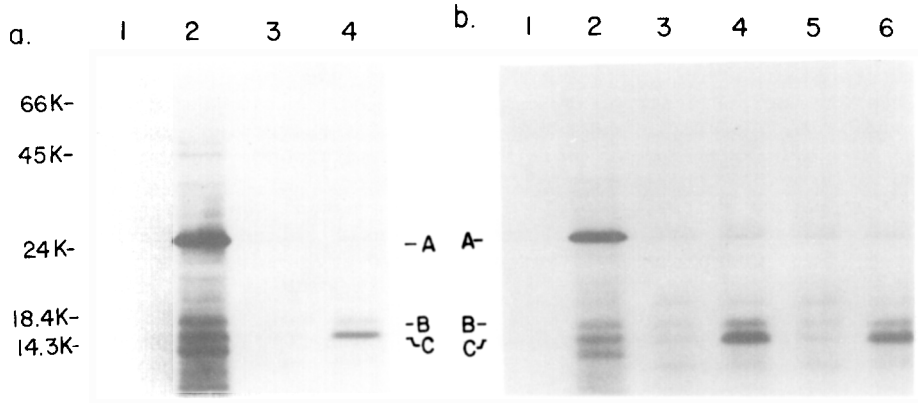

Figure 1. Immunoprecipitation of products of translation of first trimester human placenta RNA using antiserum to RCM bLH_α. Translations were performed using wheat germ extract. Messenger RNA extraction and translation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography were performed as described under "Experimental Procedures". a)Lane 1, no mRNA added; lane 2, first trimester placenta mRNA; immunoprecipitation of translation products by: lane 3, nonimmune rabbit serum;lane 4, antiserum to RCM bLH_α; b)Lane 1, no mRNA added; lane 2, first trimester placental mRNA; immunoprecipitation of translation products by: lane 3, nonimmune rabbit serum; lane 4, antiserum to RCM bLH_α; lane 5, antiserum to RCM bLH_α in the presence of excess unlabeled hCG_α; lane 6, antiserum to RCM bLH_α in the presence of excess unlabeled hCG_β. Arrows denote: A)pre-human placental lactogen; B) large pre-hCG_α subunit; C) small pre-hCG_α subunit. Apparent molecular weights were determined by co-electrophoresis with known protein standards: Bovine serum albumin, M_r=66,000; ovalbumin, M_r=45,000; trypsinogen, M_r=24,000; β-lactoglobulin, M_r= 18,400; lysozyme, M_r=14,300.

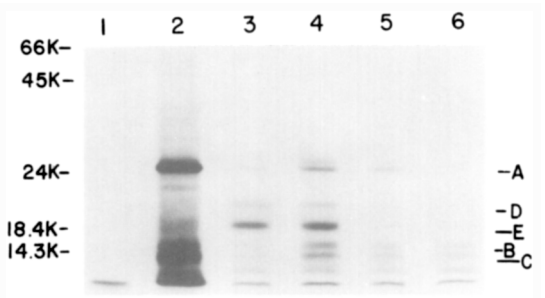

Figure 2. Immunoprecipitation of products of translation of first trimester human placenta RNA using antiserum to hCG$_\beta$. Translation were performed using wheat germ extract. Lane 1, no mRNA added; lane 2, first trimester placenta mRNA. Immunoprecipitation of translation products by: lane 3, antiserum to hCG$_\beta$; lane 4, antiserum to hCG$_\beta$ in the presence of excess cold hCG$_\alpha$; lane 5, antiserum to hCG$_\beta$ in the presence of excess cold hCG$_\beta$; lane 6, nonimmune rabbit serum. Arrows denote: A) pre-human placental lactogen; D)large pre-hCG$_\beta$ subunit; E)small pre-hCG$_\beta$ subunit; B) large pre-hCG$_\alpha$ subunit; C) small pre-hCG$_\alpha$ subunit.

previously in our laboratory (14) and by others (18), these presumed precursor forms of the α subunit were very poorly recognized by antisera against the native, glycosylated hCG$_\alpha$ subunit, but were clearly identified by the antiserum prepared using the modified immunogen (data not shown).

Similarly, immunoprecipitation using antiserum to hCG$_\beta$ identified two products of apparent M_rs= 18,500 and 21,000 (Figure 2, lane 3). In this case, the immunoprecipitation was specifically inhibited by excess unlabeled hCG$_\beta$, but not by excess unlabeled hCG$_\alpha$ (Figure 2, lanes 5 and 4, respectively).

These immunoprecipitation results, which have been verified using several different placentas and several different preparations of wheat germ extract, identify two presumed precursor forms of the hCG$_\alpha$ and hCG$_\beta$ subunits. In each case, it is the smaller of the two forms that corresponds closely in apparent molecular weight with the subunit precursors identified by others (5). Furthermore, the smaller of the α precursors comigrates with the precursors to α subunits previously identified by us in translation products of mRNA extracted from mouse thyrotrope tumor (19) and from ovariectomized rat pituitary glands (13). This would suggest that the smaller of the presumed hCG$_\alpha$ and hCG$_\beta$ precursors do not represent truncated

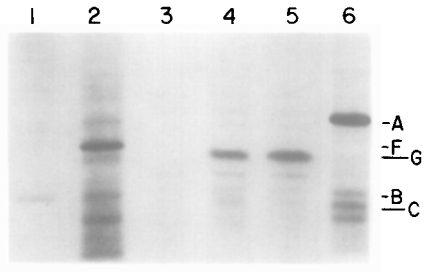

Figure 3. Immunoprecipitation of the products of translation of first trimester placenta mRNA in the presence of microsomal membranes. Translations were performed using wheat germ extract. Lane 1, no mRNA added, but microsomal membranes present; lane 2, first trimester placenta mRNA in the presence of microsomal membranes. Immunoprecipitation of membrane-supplemented translation products of placenta RNA by: Lane 3) nonimmune serum; lane 4) antiserum to RCM bLH_α; lane 5) antiserum to $hTSH_\alpha$; lane 6) translation products of first trimester placenta mRNA in the absence of microsomal membranes. Arrows denote: A) pre-human placental lactogen; F) human placental lactogen; G) glycosylated form of hCG_α; B) large pre-hCG_α subunit; C) small pre-hCG_α subunit.

forms resulting from premature termination of nascent polypeptides during the cell-free translation.

When the placental mRNA is translated in the wheat germ system supplemented with EDTA-stripped pancreatic microsomes, immunoprecipitation using antisera to RCM bLH_α or to hCG_α identifies a single band with apparent molecular weight 21,000 daltons, as noted by others (6,7) (Figure 3). It appears that either both the 14,500 dalton and the 16,000 dalton hCG_α precursors are processed to this form, or that one of them is processed to the 21,000 dalton band and the other to a product which is no longer recognized by these antisera to the α subunit. We have not yet identified a membrane-processed form of hCG_β.

The cell-free translation system derived from wheat germ, unsupplemented by microsomal membranes, is not capable of cleavage of signal sequences or of glycosylation of nascent glycoproteins (20). To verify that the larger of each pair of subunit precursors had not been glycosylated with the usual asparagine-bound core carbohydrate side chain, we digested the translation products with endo-β-acetylglycosaminidase H (endo H) an enzyme capable of cleaving specific carbohydrate side chains by splitting the

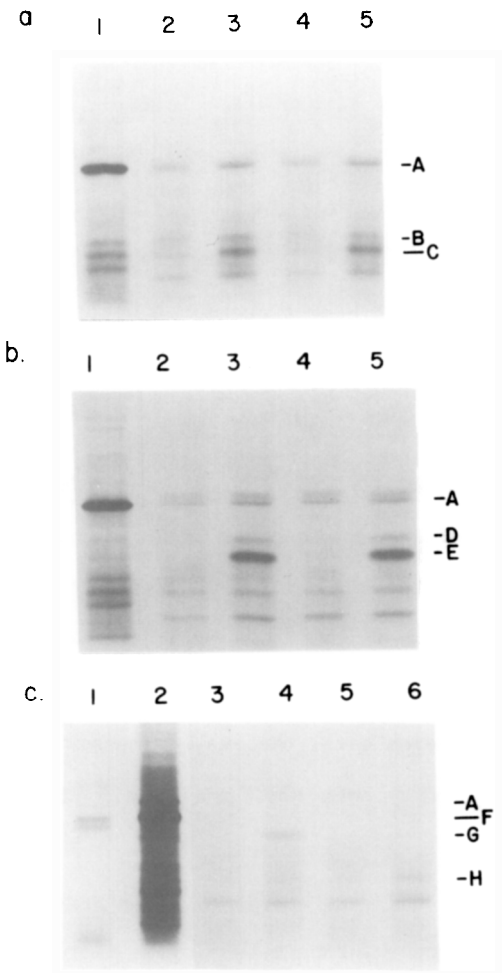

Figure 4. Immunoprecipitation of the subunits of hCG before and after digestion with endo H. Translations were performed using wheat germ extract. Endo H digestions were performed as described under "Experimental Procedures". a)No membranes; α subunit. Lane 1) translation products of first trimester placenta mRNA in the absence of microsomal membranes. Immunoprecipitation by: lane 2) nonimmune serum; lane 3) antiserum to RCM bLH_{α}; lane 4) nonimmune serum, following endo H treatment of translation products lane 5)antiserum to RCM bLH_{α}, following endo H treatment of translation products. b)No membranes; β subunit. Lane 1) translation products of first trimester placenta mRNA in the absence of microsomal membranes. Immunoprecipitation by: lane 2) nonimmune serum; Lane 3) antiserum to hCG_{β}; lane 4) nonimmune serum, following endo H treatment of translation products; lane 5) antiserum to hCG_{β}, following endo H treatment of translation products. c) With microsomal membranes; α subunit. Lane 1) no mRNA added; lane 2) translation products of first trimester mRNA in the presence of microsomal membranes. Immunoprecipitation by: Lane 3) nonimmune serum; Lane 4) anitserum to RCM bLH_{α}; lane 5) nonimmune serum, following endo H treatment of translation products; lane 6) antiserum to RCM bLH_{α}, following endo H treatment of translation products. Arrows denote: A) pre-human placental lactogen; B) large pre-hCG_{α} subunit; C) small pre-hCG_{α} subunit; D) large pre-hCG_{β} subunit; E) small pre-hCG_{β} subunit; F) human placental lactogen; G) glycosylated form of hCG_{α}; H) Endo H digestion product of band G.

asparagine-bound di-N-acetylchitobiose leaving a single asparginyl-linked N-acetylglycosamine residue (21). None of the hCG_α or hCG_β precursors was sensitive to endo H (Figures 4a and 4b). However, as expected, the 21,000 dalton form of the α subunit seen in membrane-supplemented translations was sensitive to endo H, which converted it to a form with apparent molecular weight 13,000 daltons (Figure 4c). Therefore, if glycosylation accounts for the doublet of both α and β precursors, it would appear that the sugar moieties are not the characteristic asparagine-bound core carbohydrates.

DISCUSSION

Using immunoprecipitation analysis, we have identified two cell-free translation products of human placental mRNA having structural homology with the α subunit of the glycoprotein hormones. These products have apparent M_rs = 14,500 and 16,000. In addition, we have identified two other products with apparent M_rs = 18,500 and 21,000 that have structural homology with hCG_β. In each case, the smaller member of the doublet corresponds to the subunit precursor previously identified by other investigators.

Experience with the wheat germ system, resistance to endo H digestion, and susceptibility to processing by heterologous microsomal membranes would suggest that neither member of the doublet is glycosylated. It would therefore appear that there are two precursor forms for each of the subunits of hCG.

The explanation for our observation of two forms of each of the subunits in the wheat germ cell-free system is open to speculation at present. Furthermore, it is not known whether the two α subunit precursors result from the same mechanism which generates the two β subunits precursors. The most provocative possibility is that, for one or both subunits, the two forms are translated from separate mRNA molecules representing separate genes. Alternatively, the two forms of the subunit precursors may be translation products of two separate mRNA molecules arising from variation

in the splicing of a single, larger mRNA precursor transcribed from a single gene.

In the former circumstance, we would expect there to be several copies of hCG subunit-related genes in the human genome. The structural similarities among the β subunits of the various glycoprotein hormones and even between the α and β subunits of the hormones (1-3) suggests the possibility that these genes have arisen by duplication of an ancestral gene. Analysis of restriction endonuclease digests of human placental DNA (11,22,23) and of DNA from several hCG-producing tumor cell lines (22) has been interpreted to demonstrate the existence of a single gene for hCG_{α}. However, this evidence, although very suggestive, does not entirely exclude the possibility of multiple α subunits genes. No data has yet been presented bearing on the structure or multiplicity of genes for hCG_{β}.

The latter circumstance, namely utilization of alternative splicing patterns in the processing of a single mRNA precursor, may be a more tenable explanation for our observations in light of evidence for a single α subunit gene. Such as explanation has been persuasively advanced for the existence of two forms of human growth hormone (24) and of two tissue-specific forms of mouse α-amylase mRNA differing in their 5' noncoding regions (25). The frequency of occurrence and biological importance of such splicing variants remain to be explored.

It is possible that, for one or both subunits, the two forms are translated from the same mRNA molecule, with ribosomal binding and chain initiation occurring at two different points. For example, initiation might occur at two different AUG codons, resulting in signal sequences of different length. This would be at odds with the suggestion that initiation occurs at the AUG codon closest to the 5' end of the mRNA (26), but might occur as a result of the particular conditions of cell-free translation. It would explain the observation that the two forms of hCG_{α} appear to be processed to a single 21,000 dalton band by microsomal membranes,

since the heterogeneity would reside entirely in the cleaved N-terminal leader sequences.

However, this possibility is extremely unlikely to explain the existence of two α subunit precursors, since the entire 5' noncoding region of the mRNA for the α subunit has been determined and contains no additional AUG codons (23). The 5' noncoding region of the mRNA for the β subunit remains to be fully defined (12), and so this explanation remains theoretically viable in that case.

Our data do not exclude the possibility that the larger of the two forms of one or both subunits contains a small amount of endo H-resistant carbohydrate, though this would be quite unexpected. It is also possible that the doublets result from specific coprecipitation of unrelated proteins with the subunit precursors, but this phenomenon has not been observed by us in other systems and would seem unlikely to occur in the denaturing conditions under which our immunoprecipitations were performed.

The explanation for the observation of two distinct $hCG\alpha$ and two distinct $hCG\beta$ precursors is not yet evident. However, the cDNAs encoding these subunits are available and may be utilized to explore this problem further. We expect to have a more complete understanding of the organization of the genes encoding the subunits of the glycoprotein hormones in the near future.

ACKNOWLEDGEMENTS

We thank Dr. J.G. Pierce for generously providing antiserum to reduced and carboxymethylated bovine LH_α, Dr. P.W. Robbins for kindly providing Endo-β-N-acetylglucosaminidase H, Dr. J. Majzoub for isolating and providing dog pancreas microsomes, and Ms. Jeanne Sullivan and Ms. Janice Blades for preparing the manuscript.

REFERENCES

1. Pierce, J.G., Liao, T.H., Howard, S.M., Shome, B., and Cornell, J.S. (1971) Recent Prog. Horm. Res. 27, 165-212.
2. Vaitukaitis, J.L., Ross, G.T., Braunstein, G.D., and Rayford, P.L. (1976) Recent. Prog. Horm. Res. 32, 289-332.

3. Ward, D.N., Reichart, L.E., Jr., Liu, W.K., Nahm, H.S., Hsia, J., Lamkin, W.M., and Jones, M.D. (1973) Recent Prog. Horm. Res. 29, 533-562.
4. Landefeld, T., Boguslawski, S., Corash, L., and Boime, I. (1976) Endocrinology 98, 1220-1227.
5. Daniels-McQueen, S., McWilliams, D., Birken, S., Canfield, R., Landefeld, T., and Boime, I. (1978) J. Biol. Chem. 253, 7109-7114.
6. Bielinska, M., and Boime, I. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1768-1772.
7. Bielinska, M., Grant, G.A., and Boime, I. (1978) J. Biol. Chem. 253, 7117-7119.
8. Ruddon, R.W., Hanson, C.A., and Addison, N.J., (1979) Proc. Natl. Acad. Sci.U.S.A. 76, 5143-5247.
9. Ruddon, R.W., Hanson, C.A., Bryand, A.H., Putterman, G.J., White, E.L., Perini, R., Meade,K.S., and Aldenderfer, P.H., (1980) J. Biol. Chem. 256, 5189-5196.
10. Ruddon, R.W., Bryan, A.H., Hanson, C.A., Perini, F., Ceccorulli, L.M., and Peters, B.P. (1981) J. Biol. Chem. 256, 5189-5196.
11. Fiddes, J.C., and Goodman, H.M. (1979) Nature 281, 351-356.
12. Fiddes, J.C., and Goodman, H.M. (1980) Nature 286, 684-687.
13. Godine, J.E., Chin, W.W., and Habener, J.F. (1980) J. Biol. Chem. 255, 8780-8783.
14. Godine, J.E., Chin, W.W., and Habener, J.F. (1981) J. Biol. Chem. 256, 2475-2479.
15. Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G., and Lodish, H. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3278-3282.
16. Boime, I., McWilliams, D., Szczesna, E., and Camel, M. (1976) J. Biol. Chem. 251, 820-825.
17. Cox, G.S., Weintraub, B.D., Rosen, S.W., and Maxwell, E.S., (1976) J. Biol. Chem. 251, 1723-1730.
18. Giudice, L.C., and Weintraub, B.D., (1979) J. Biol. Chem. 254, 12679-12683.
19. Chin, W.W., Habener, J.F., Kieffer, J.D., and Maloof, F. (1978) J. Biol. Chem. 253, 7985-7988.
20. Roberts, B.E., and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2330-2334.
21. Tarentino, A.L., Plummer, T.H., Jr., and Maley, F. (1974) J. Biol. Chem. 249, 818-824.
22. Boothsby, M., Ruddon, R.W., Anderson, C., McWilliams, D., and Boime, I. (1981) J. Biol. Chem. 256, 5121-5127.
23. Fiddes, J.C., and Goodman, H.M., (1981) J. Mol. Appl. Gen. 1, 3-18.
24. Cooke, N.E., Bacta, A., Cathala, G., Richards, R., Spindler, S.S., Mellon-Nussbaum, S., Shine, J., and Baxter, J.D. (1981) Clin. Res. 29, 504A.
25. Young, R.A., Hagenbiichle, O., and Schibler, U., (1981) Cell 23, 451-458.
26. Kozak, M. (1980) Cell 22, 7-8.