Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2432–2436, June 1977
Cell Biology

Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin

(mRNA translation/wheat germ extract/microsomal membranes/cleavage and segregation/radiosequencing)

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Communicated by Severo Ochoa, March 14, 1977

ABSTRACT **Major translation products of bovine pituitary RNA in a wheat germ cell-free system were identified as larger forms (prehormones) of growth hormone and prolactin containing amino-terminal extensions of 26 or 27 and 30 amino acid residues, respectively. However, translation of bovine pituitary RNA in the wheat germ cell-free system in the presence of microsomal membranes prepared from canine pancreas or bovine pituitary yielded products that were of the same size as authentic growth hormone and prolactin; by partial amino-terminal sequence analysis they were shown to contain the correct unique amino-terminal sequence of prolactin and the two correct amino termini of authentic growth hormone; moreover, they were found to be segregated within the microsomal vesicles in that they were largely inaccessible to degradation by proteolytic enzymes. When microsomal membranes were present *after* rather than *during* translation, prehormones were neither cleaved nor segregated. These results strongly suggest that the synthesis and segregation of the authentic hormone observed in the presence of membranes proceeds via a *nascent* prehormone rather than a *completed* prehormone.**

Several laboratories have reported on the biosynthesis *in vitro* of the pituitary hormones prolactin (1–3) and growth hormone (4). Cultured pituitary tumor cells or rat pituitaries were used in those studies as sources for the extraction of a total mRNA fraction which was then translated in a heterologous system. Immunoprecipitation was used to search for the growth hormone and prolactin among the translation products. It was found that both hormones were synthesized as larger molecules. For the larger form of prolactin (preprolactin) it was recently established (5) that a 29-amino-acid residue extension is localized at the amino terminus.

In this paper we report our data on the biosynthesis of prolactin as well as of growth hormone *in vitro* using readily available bovine pituitaries as a source of mRNA. Our experiments were designed to investigate the biosynthetic relationship between the larger and the authentic forms of these two hormones.

METHODS

Fractionation of Bovine Anterior Pituitary. Fresh bovine pituitaries were obtained from a local abattoir. Dissected anterior lobes were minced and homogenized in 2 volumes of ice-cold 0.25M sucrose. The homogenate was centrifuged 10 min at 1000 × *g* to yield a postnuclear supernate. A portion of the postnuclear supernate was centrifuged for 10 min at 12,000 × g_{av} in an angle rotor. The resulting postmitochondrial supernate was used to prepare rough microsomes (6). Microsomal membranes were prepared from rough microsomes using EDTA for the removal of ribosomes (7).

RNA Extraction. A portion of the postnuclear supernate was used to isolate RNA by phenol/chloroform/isoamyl alcohol as described (6).

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

Cell-Free Protein Synthesis. A wheat germ S-23 cell-free system was prepared by the method of Roman *et al.* (8). Incubations were performed in a final volume of 100 μl, as described (9), and contained 20 μCi of [^{35}S]methionine and 0.7 A_{260} unit of RNA. Incubations were at 27° for 90 min. Dog pancreas microsomal membranes were prepared from rough microsomes using EDTA for the removal of ribosomes (7).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate ($NaDodSO_4$). Trichloroacetic acid precipitates or immunoprecipitates were reduced and alkylated as described (6). Electrophoresis of the reduced and alkylated samples in polyacrylamide gradient slab gels in $NaDodSO_4$ and subsequent autoradiography of the dried slab gels was as described (6).

Partial Sequence Determination. Incubation volumes for protein synthesis were scaled up; they contained [^{35}S]methionine and tritiated amino acids. The translation products were subjected to electrophoresis (see above). However, the slab gels were dried directly onto Whatman 3 MM paper after the conclusion of electrophoresis without prior staining. After autoradiography, usually for 1–2 days, the desired band was excised from the dried gel and the radioactivity was eluted by shaking at room temperature in electrophoresis buffer [Tris-glycine (10) in 0.1% $NaDodSO_4$]. The eluant was dialyzed for 24 hr with 3 mg of carrier myoglobin against water, concentrated to less than 5 ml by lyophilization, precipitated with 9 volumes of acetone, resuspended in heptafluorobutyric acid, and then subjected to automated Edman degradation in the Beckman 890C Sequencer using an improved DMAA program (no. 102974). Up to 60 cycles of Edman degradations were carried out. The thiazolinones were converted to phenylthiohydantoin amino acids.

All data (except for those of Fig. 7) have been presented uncorrected for background or for specific activity differences of the various radioactive amino acids.

Sources of Materials. [^{3}H]Alanine (12 Ci/mmol), [^{3}H]asparagine (5 Ci/mmol), [^{3}H]leucine (57.4 Ci/mmol), [^{3}H]phenylalanine (40 Ci/mmol), [^{3}H]proline (60 Ci/mmol), and [^{3}H]threonine (1.8 Ci/mmol) were from New England Nuclear, Boston, MA. [^{35}S]Methionine (580 Ci/mmol) was from Amersham-Searle, Arlington Heights, IL. Sperm whale apomyoglobin was obtained from Beckman Instruments, Inc., Palo Alto, CA. Rabbit antiserum to ovine prolactin was from Calbiochem, San Diego, CA. Sheep antiserum to rabbit F(ab′)$_2$ was a kind gift of S. Silverstein.

RESULTS

RNA extracted from the anterior lobe of bovine pituitary resulted in a 15 to 20-fold stimulation of amino acid incorporation when added to a wheat germ cell-free system (data not shown). Analysis of the translation products by polyacrylamide gel electrophoresis in $NaDodSO_4$ and subsequent autoradiography of the dried gel yielded two major bands with apparent mo-

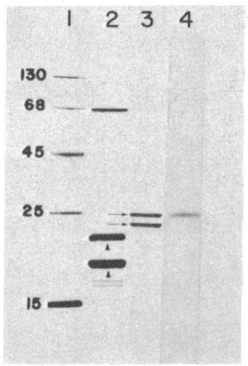

FIG. 1. Characterization of polypeptides synthesized by translation of anterior pituitary RNA in the wheat germ cell-free system. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis. Bands in lanes 1 and 2 are caused by Coomassie brilliant blue stain; those in lanes 3 and 4 are from an autoradiograph. Lane 1: molecular weight standards: *Escherichia coli* β-galactosidase (130,000), bovine serum albumin (68,000), ovalbumin (45,000), porcine chymotrypsinogen (25,000), and rabbit globin (15,000). Lane 2: anterior pituitary homogenate. Lane 3: translation products of anterior pituitary RNA. Lane 4: immunoprecipitate of translation products using an antiserum to prolactin. Immunoprecipitation was as described (11). Arrowheads (lane 2) point to bands with a mobility identical to prolactin (upper) and growth hormone (lower). Horizontal arrows point to preprolactin (upper) and pregrowth hormone (lower).

lecular weights of 25,000 and 24,000 (Fig. 1, lane 3). A corresponding preponderance of two polypeptides with apparent molecular weights of 22,000 and 20,000 was observed when the entire homogenate of anterior pituitary was analyzed (Fig. 1, lane 2). The latter two bands were tentatively identified as prolactin (22,000-dalton band) and growth hormone (20,000-dalton band) on the basis of comigration with commercially obtained hormones (data not shown). On the assumption that the abundance of proteins in the homogenate might reflect the abundance of translatable mRNAs, we tentatively identified the two major translation products of bovine anterior pituitary mRNA (Fig. 1, lane 3) as larger forms of prolactin (25,000-dalton band) and of growth hormone (24,000-dalton band). This assignment was supported by immunoprecipitation of the translation products with an antibody against ovine prolactin: only the 25,000-dalton polypeptide was precipitated (Fig. 1, lane 4). These tentative identifications were shown to be correct by the sequence data described below.

From sequence data now available for several presecretory proteins synthesized *in vitro* (5, 12–15) and from the predictions made in the signal hypothesis (6) it could be anticipated that the two larger forms of bovine prolactin and growth hormone contain the sequence of the respective authentic hormones plus a characteristic amino-terminal sequence extension. This assumption was verified (Figs. 2 and 3) by subjecting the radioactively labeled molecules to automated sequential Edman degradations. For the 24,000-dalton band, which was synthesized in the presence of [^{35}S]methionine and several tritiated amino acids (leucine, proline, and phenylalanine), liquid chromatography was used (Devillers-Thiery and Blobel, in preparation) after each Edman degradation cycle in order to identify the phenylthiohydantoin derivatives of the labeled amino acids. The discrete peaks of radioactivity (Fig. 2) enabled us to identify leucine as the amino acid at residues 10, 11, 12, 16, 17, 19, and 33; proline at residues 6, 20, and 29; phenylalanine at residues 14 and 28; and methionine at residues 1, 2, and 31. The identification of Phe_{28}, Pro_{29}, Met_{31}, and Leu_{33} permitted alignment (Fig. 4) with the amino-terminal sequence of authentic bovine growth hormone and thus provided strong

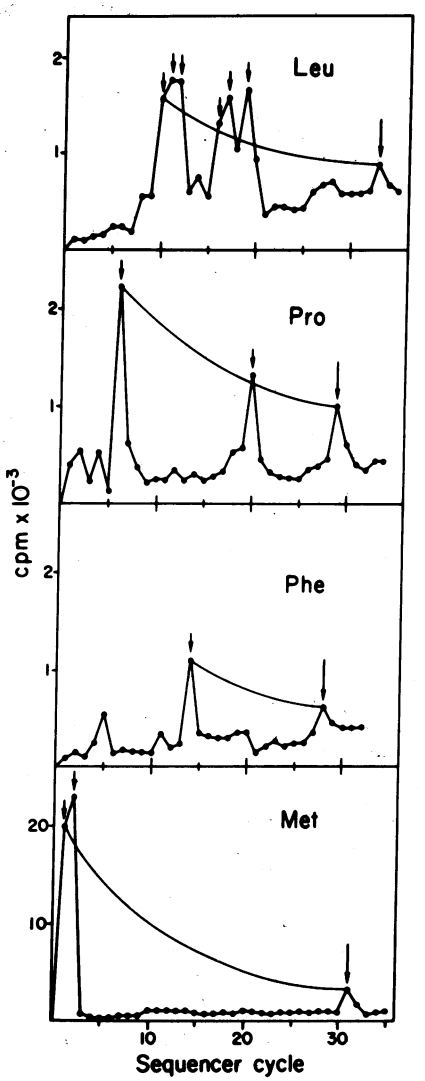

FIG. 2. Partial amino-terminal sequence analysis of pregrowth hormone. Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing 100 μCi of [^{3}H]leucine, 100 μCi of [^{3}H]proline, 60 μCi of [^{3}H]phenylalanine, and 400 μCi of [^{35}S]methionine in 2 ml final volume. The pregrowth hormone band (lower of the horizontal arrows in lane 3 of Fig. 1) was taken through 35 cycles of Edman degradation. The curved line indicates the theoretical yield of radioactivity based on the calculated repetitive yield (16) normalized to the first radioactive peak. Sequence positions in the signal peptide region are indicated by short arrows, those in the authentic region by long arrows.

evidence that the 24,000-dalton band is pregrowth hormone in which the amino terminus of authentic growth hormone is preceded by 26 or 27 (see below) amino acid residues.

For the 25,000-dalton band, which was labeled with [^{35}S]-methionine and only one tritiated amino acid (proline), double labeling counting procedures after each Edman degradation cycle were sufficient to identify methionine and proline. From the peaks of radioactivity (Fig. 3) proline was identified as the amino acid at residues 32, 35, and 38 and methionine at residues 1 and 54. The identification of Pro_{32}, Pro_{35}, Pro_{38}, and Met_{54} permitted alignment with the authentic amino-terminal sequence of bovine prolactin (Fig. 4). Together with the immunological identification, these data provide strong evidence that the 25,000-dalton band is preprolactin. In this case the amino terminus of authentic prolactin is preceded by 30 amino acid residues.

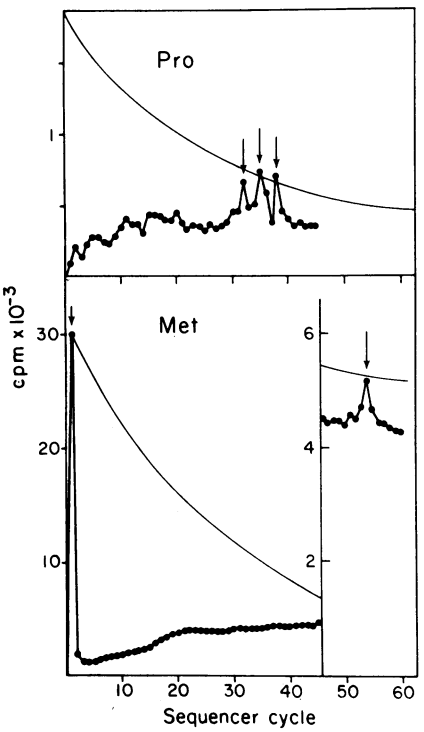

FIG. 3. Partial amino-terminal sequence analysis of preprolactin. Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing 100 μCi of [^{3}H]proline and 400 μCi of [^{35}S]methionine in 2 ml final volume. The preprolactin band (upper of the horizontal arrows in lane 3 of Fig. 1) was taken through 60 cycles of Edman degradation. Curved line and arrows as in Fig. 2.

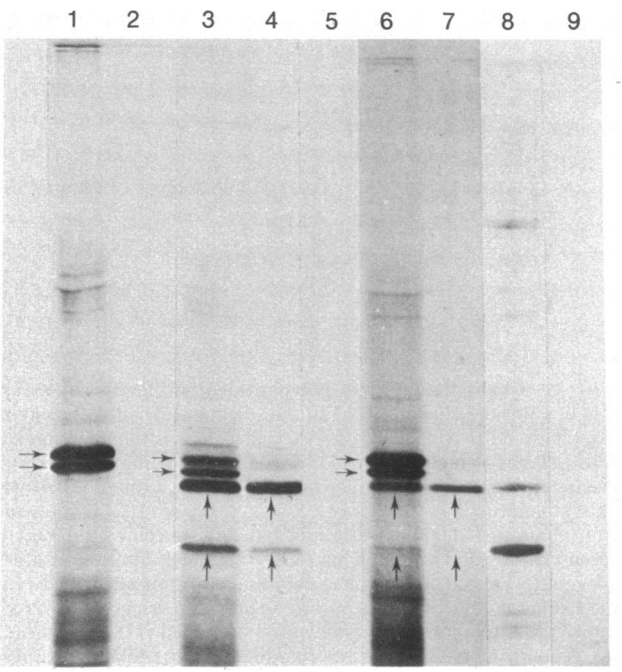

FIG. 5. Cleavage and segregation of nascent bovine preprolactin and pregrowth hormone *in vitro* by microsomal membranes. Analysis was by NaDodSO$_4$/polyacrylamide gel electrophoresis and staining only (lanes 8 and 9) or subsequent autoradiography of dried slab gels (lanes 1–7). Bovine anterior pituitary RNA was translated in the wheat germ cell-free system. Lane 1: posttranslational incubation for 90 min at 27° with dog pancreas microsomal membranes (2.5 A_{260} units/ml); lane 2: as in lane 1, but followed by incubation with proteolytic enzymes; lane 3: dog pancreas microsomal membranes (2.5 A_{260} units/ml) were present during translation; lane 4: as in lane 3, but followed by incubation with proteolytic enzymes; lane 5: as in lane 4, except that proteolysis was in the presence of 0.5% sodium deoxycholate; lane 6: bovine pituitary microsomal membranes (5.0 A_{260} units/ml) were present during translation; lane 7: as in lane 6, but followed by incubation with proteolytic enzymes; lane 8: high-speed supernate (1 hr, 100,000 × g_{av}) of pituitary homogenate; lane 9: as in lane 8, but incubated with proteolytic enzymes. Incubation with proteolytic enzymes (200 μg each of trypsin and chymotrypsin per ml) was for 60 min at 27°. Horizontal arrows point to preprolactin (upper) and pregrowth hormone (lower). Vertical arrows point to their "processed" forms: prolactin (upper) and growth hormone (lower) with a mobility identical to their presumptive counterparts present in a pituitary homogenate (lane 8).

It has been shown that translation *in vitro* of mRNAs for secretory proteins in the presence of microsomal membranes resulted in reconstitution of functional rough microsomes from heterologous components. Reconstitution was inferred from the fact that most of the translation products were "processed," i.e., were shortened to the size of authentic proteins (7, 17), and were "segregated," i.e., were resistant to posttranslational proteolysis (7). Translation of bovine anterior pituitary RNA in the wheat germ cell-free system (Fig. 5) but in the presence of microsomal membranes from either dog pancreas (lane 3) or bovine pituitary (lane 6) resulted in the appearance of two new, faster moving bands (indicated by vertical arrows) that

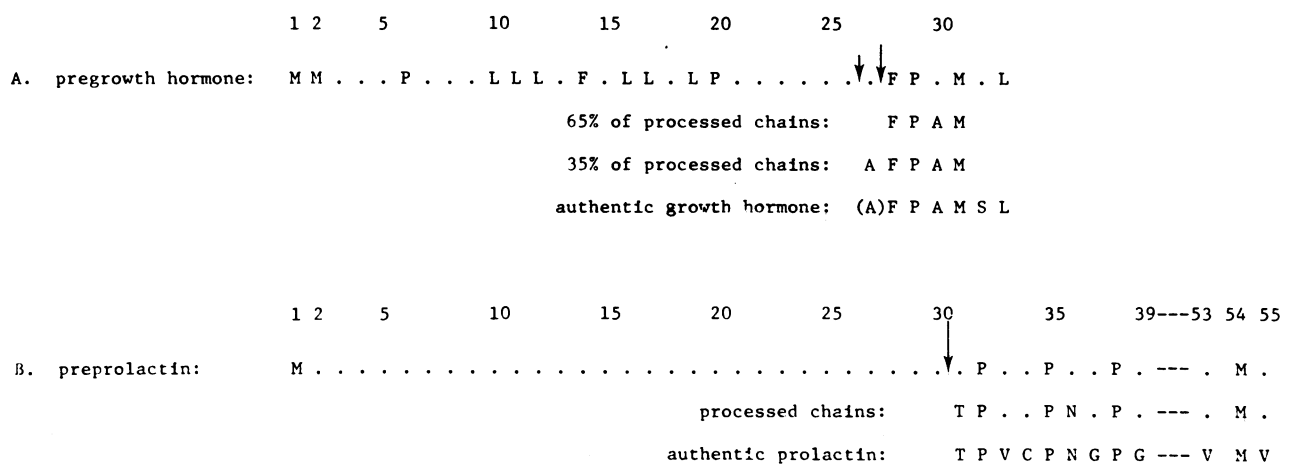

FIG. 4. Summary of radiosequence data of (*A*) pregrowth hormone and growth hormone and (*B*) preprolactin and prolactin synthesized *in vitro* (see Figs. 2, 3, 6, and 7). Arrows indicate cleavage site for signal peptidase. A, Ala; C, Cys; F, Phe; G, Gly; L, Leu; M, Met; N, Asn; P, Pro; S, Ser; T, Thr; V, Val.

		1	2	3	4	5	6	7	8	9 ... 23	24	25
A. authentic bovine prolactin		Thr	Pro	Val	Cys	Pro	Asn	Gly	Pro	Gly...Val	Met	Val
B. processed chain: pituitary membranes	[^{3}H] Pro	112	750	169	220	450	187	173	435	151		
C. processed chain: pancreatic membranes:	[^{3}H] Pro	80	1,850	360	240	2,450	390	265	1,940	n.d.		
D. processed chain: pancreatic membranes; protected from proteolysis	[^{35}S] Met	63	21	14	16	17	n.d.	16	20	22... 33	273	85
	[^{3}H] Pro	24	297	43	48	274	72	37	175	52		
	[^{3}H] Thr	28	15	n.d.	10	14	12	10	14	14		
	[^{3}H] Asn	34	35	n.d.	36	24	96	32	14	17		

FIG. 6. Partial amino-terminal sequence analysis of prolactin synthesized *in vitro* and alignment with the amino-terminal sequence of authentic bovine prolactin (*A*). Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing in 2 ml final volume: (*B*) 100 μCi of [^{3}H]proline, 400 μCi of [^{35}S]methionine, and 10.0 A_{260} units of microsomal membranes from bovine anterior pituitary; (*C*) as *B* except that the microsomal membranes (5.0 A_{260} units) were from dog pancreas; (*D*) 100 μCi of [^{3}H]proline, 50 μCi of [^{3}H]threonine, 80 μCi of [^{3}H]asparagine, 400 μCi of [^{35}S]methionine, and 5.0 A_{260} units of dog pancreas microsomal membranes. In this case translation was followed by incubation with proteolytic enzymes (see Fig. 5). The prolactin band (upper of the vertical arrows in Fig. 5) was taken through 9–25 cycles of Edman degradation. The data shown are cpm, with assigned positions underlined. n.d., not determined.

had apparent molecular weights of 22,000 and 20,000 and comigrated with authentic prolactin and growth hormone.

These results (Fig. 5) suggested that translation in the presence of microsomal membranes, heterologous or homologous with respect to the mRNA, resulted in "processing," which was more (lane 3) or less (lane 6) complete. Moreover, the processed chains were resistant to subsequent proteolysis by added trypsin and chymotrypsin presumably because they were segregated in the lumen of the membrane vesicles (lanes 4 and 7). In contrast, the remaining unprocessed chains (lanes 3 and 6, horizontal arrows) were degraded (lanes 4 and 7) because they were presumably not segregated. However, after solubilization of the protecting microsomal membrane by detergent, even the processed chains were sensitive to proteolysis (lane 5). Furthermore, polypeptides present in a high speed supernatant (1 hr at 100,000 × *g*) of a bovine anterior pituitary homogenate (lane 8) were digested (lane 9) during proteolysis in the absence of detergent. Thus, sensitivity to proteolysis was not due to detergents *per se*, nor was protection from proteolysis an intrinsic property of the authentic hormone. Most likely, therefore, it was a result of localization within membranous vesicles. Processing and segregation only occur when the microsomal membranes are present *during* translation. Posttranslational incubation with microsomal membranes did not result in processing (lane 1), i.e., there were no bands in the position of the authentic hormones, nor does it result in segregation, i.e., there was no protection from proteolytic enzymes (lane 2).

The remaining question of interest was to investigate the fidelity of processing. To this intent we determined the amino-terminal sequence (Figs. 6 and 7) of the processed chains that resulted from translation of RNA in the presence of either heterologous or homologous microsomal membranes. The sequence data that were obtained showed that the two processed chains resulted from a *bona fide* cleavage since their partially determined amino-terminal sequence was identical to that of the authentic prolactin and growth hormone, respectively (Fig. 4).

Thus, it was found (Fig. 6) that the 22,000 band (Fig. 5, lanes 3 and 6), synthesized in the presence of [^{35}S]methionine and [^{3}H]proline, contained proline at residues 2, 5, and 8 regardless of whether homologous (Fig. 6*B*) or heterologous (Fig. 6*C*) membranes were used for processing. Likewise, when synthesized in the presence of [^{35}S]methionine and three tritiated amino acids (proline, threonine, and asparagine) but in addition subjected to posttranslational proteolysis by trypsin and chymotrypsin (Fig. 5, lane 4), the 22,000-dalton band contained

		1 Phe Ala	2 Pro Phe	3 Ala Pro	4 Met Ala	5 Ser Met	Ser
A. authentic bovine growth hormone							
B. processed chain: pancreatic membranes	[^{3}H] Ala:	23	8	32	22	8	10
	[^{3}H] Phe:	110	70	17	0	5	20
	[^{3}H] Pro:	27	173	110	40	36	n.d.
	[^{35}S] Met:	205	60	55	1,440	1,060	160

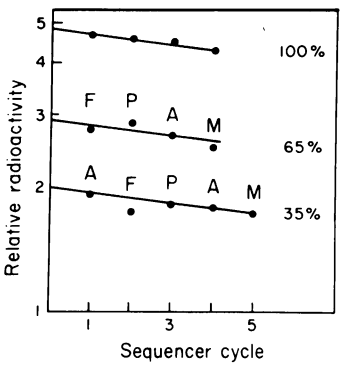

FIG. 7. (*Left*) Partial amino-terminal sequence analysis of growth hormone synthesized *in vitro* and alignment with the amino-terminal sequence of the two forms of authentic bovine growth hormone (*A*), differing in the absence or presence of an initial alanine (18). Bovine anterior pituitary RNA was translated in a wheat germ cell-free system containing in 2 ml final volume: 50 μCi of [^{3}H]alanine, 60 μCi of [^{3}H]phenylalanine, 100 μCi of [^{3}H]proline, 400 μCi of [^{35}S]methionine, and 5.0 A_{260} units of dog pancreas microsomal membranes. The growth hormone band (lower of the vertical arrows in Fig. 5) was taken through 5 cycles of Edman degradation. The data shown are cpm with assigned positions underlined (see *text*). (*Right*) Heterogeneity of growth hormone synthesized *in vitro* with respect to amino-terminal residue. Data shown at *left* were converted into arbitrary units of relative radioactivity (cpm divided by specific activity of radioactive amino acid). Note logarithmic ordinate. The uppermost line (100%) represents the calculated sum of the relative radioactivity of two amino acid residues released in each cycle of the sequence. The lower lines represent the relative radioactivities of individual residues in each cycle (see *text*).

(Fig. 6*D*) proline at residues 2, 5, and 8, threonine at residue 1, asparagine at residue 6, and methionine at residue 24.

For the 20,000-dalton band (Fig. 5, lane 3), synthesized in the presence of [^{35}S]methionine and three tritiated amino acids (phenylalanine, proline, and alanine), the distribution of radioactivity (Fig. 7 *left*) was not confined to single cycle peaks (as, for instance, for the 22,000-dalton band, see Fig. 6); instead, each peak was followed by a shoulder in the subsequent cycle. When peak and shoulder radioactivities were expressed as "relative" radioactivities (i.e., cpm divided by specific activity of respective amino acid) and plotted separately (Fig. 7 *right*), it became evident that the 20,000-dalton band consisted of two discrete populations of chains, which differed from each other by the presence or absence of an initial alanine. This heterogeneity is similar to that found in authentic bovine growth hormone preparations (18). Thus, identification of the processed chains as authentic bovine growth hormone was made on the basis of alignment of Ala_1, $Phe_{1/2}$, $Pro_{2/3}$, $Ala_{3/4}$, and $Met_{4/5}$.

CONCLUSION

Consistent with earlier studies on rat prolactin (1–3, 5) and growth hormone (4), the data presented here demonstrate that the translation products of mRNA coding for bovine growth hormone and prolactin are larger molecules containing amino-terminal extensions of 26 or 27 and 30 amino acid residues, respectively (see Fig. 4). More significantly, however, we have been able to define the conditions under which the authentic rather than the larger form of these two hormones can be synthesized and segregated in a cell-free system. This, in fact, amounts to a reconstruction *in vitro* of the initial steps of the secretory pathway (19) compatible with the sequence of events outlined in the recently proposed signal hypothesis (6).

Particularly remarkable was the fidelity of cleavage, which converted *nascent* preprolactin into *nascent* prolactin. Even more intriguing was the cleavage of *nascent* bovine pregrowth hormone. The authentic hormone occurs in two forms which differ by the presence or absence of an initial alanine (18). Thus, the faithful reproduction *in vitro* of this heterogeneity by a heterologous signal peptidase strongly suggests that the cleavage site in *nascent* pregrowth hormone may be sufficiently ambiguous so as to result in random cleavage either before or after alanine. Fidelity of cleavage has recently been observed also with nascent fish preproinsulin (Shields and Blobel, in press) and with nascent human placental prelactogen (20).

It should be noted that cleavage as well as segregation only operates on *nascent* not on *completed* chains, i.e., it occurs only when membranes are present *during* but not *after* translation (see Fig. 5). This, together with other previously presented evidence (6, 7), implies that it is not the larger completed molecules (synthesized *in vitro* in the absence of membranes and often referred to as "precursors"), but rather their uncompleted nascent forms that are the physiological intermediates in the synthesis of authentic secretory proteins.

We thank B. Dobberstein for his participation in the initial stages of this work and H. Desruisseaux for technical assistance. This work was supported by Grant CA 12413 from the National Cancer Institute.

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