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Pre-proparathyroid Hormone: A Direct Translation Product of Parathyroid Messenger RNA

(wheat germ extract/tryptic peptides/precursor/cyanogen bromide cleavage)

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ABSTRACT **An 8–15S RNA fraction from calf parathyroid glands stimulated the incorporation of radioactive lysine and methionine into protein by 15- to 30-fold in a wheat germ extract. The major product, representing 25% of the total protein synthesized, could be bound to an antiserum to parathyroid hormone and binding was inhibited by parathyroid hormone. The chromatographic mobilities of the two tryptic peptides of the cell-free product that contained methionine were identical to the corresponding peptides of parathyroid hormone. Upon electrophoresis in acidic or sodium dodecyl sulfate–acrylamide gels, the cell-free product migrated more slowly than either parathyroid hormone or its biosynthetic precursor, proparathyroid hormone. Analysis of cyanogen bromide products indicated that the cell-free product contained an additional sequence of amino acids at the amino-terminal end. A protein corresponding to the cell-free product could not be detected in intact cells even during incubations with [^{3}H]leucine as short as 2 min, which suggests the protein may be a transient precursor to proparathyroid hormone.**

The information encoded in messenger RNA and translated into protein is not always a part of the ultimate structure of the protein product. This is a common phenomenon in the biosynthesis of eukaryotic proteins destined for secretion; for example, precursors have been described for polypeptide hormones, collagen, proteolytic enzymes (see ref. 1 for references) and immunoglobulins (2). The precursors are converted to their respective proteins by post-translational cleavage. The functions of the precursors are not always clear, although it has been suggested that they may facilitate the intracellular transport of the protein (3) or facilitate the folding of the protein into the proper conformation (4). In some cases the precursor is biologically inactive, and conversion of the precursor to its final form may regulate the production of the active species.

Parathyroid hormone (PTH) is initially synthesized as a precursor, proparathyroid hormone (ProPTH) (1, 5, 6). In studies in intact cells, it is not possible to demonstrate that ProPTH is the initial, direct product of the translation of messenger RNA for PTH. To determine whether the parathyroid messenger RNA might contain more information than is represented in the structure of ProPTH, we have begun studies to isolate the messenger RNA for PTH and to translate it in a heterologous cell-free system. We now report that RNA isolated from parathyroid glands can be translated in cell-free extracts of wheat germ and that the major product synthesized is slightly larger than ProPTH. The results indicate that the product, termed pre-ProPTH, may be a precursor to ProPTH.

Abbreviations: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; pre-ProPTH, pre-proparathyroid hormone; TMV, tobacco mosaic virus; TSSE buffer, 0.01 M Tris·HCl, pH 7.4, 0.5% sodium dodecyl sulfate, 0.1 M NaCl and 1 mM ethylenediaminetetraacetate.

METHODS

Isolation of RNA. Calf parathyroid glands, obtained from a local abattoir at the time of slaughter, were immediately placed in cold Earle's balanced salt solution and used within 2 hr. Approximately 1 g (wet weight) of glands was minced and homogenized with a motor-driven Teflon–glass homogenizer at 0° in 5 ml of 0.25 M sucrose, 0.01 M Tris·HCl, pH 7.4 (25°), containing 100 μg/ml of heparin. The homogenate was centrifuged at 1000 × *g* for 5 min. The supernatant was adjusted to 1.0% sodium dodecyl sulfate (Na DodSO$_4$) and 0.04 M Na ethylenediaminetetraacetate (EDTA), pH 7.4, and deproteinized by shaking three times with two volumes of phenol–chloroform–isoamyl alcohol (1:1:0.04) and finally three times with chloroform–isoamyl alcohol (1:0.04) (7). The final chloroform phase was re-extracted with 5 ml of 0.01 M Tris·HCl, pH 7.4, 0.5% Na DodSO$_4$, 0.1 M NaCl, and 1 mM EDTA (TSSE buffer). RNA was precipitated overnight at −20° by the addition of 2 volumes of ethanol to the combined aqueous phases. The RNA was dissolved in 0.4 ml of TSSE buffer and was layered over a 5–20% sucrose gradient in TSSE buffer and centrifuged at 29,000 rpm for 15 hr at 20° in a Beckman SW 41 rotor. The fraction of the gradient containing material with sedimentation coefficients between 8 S and 15 S (chosen arbitrarily) was collected, 2 volumes of ethanol were added, and the sample was left overnight at 20°. The precipitate was collected by centrifugation at 10,000 × *g* and was washed several times by dissolving in water and reprecipitating with ethanol at −20° for at least 2 hr. The RNA was redissolved in water and stored at −20°. The amount of RNA was estimated from the absorbance at 260 nm (25 $A_{260\,nm}$/mg). Tobacco mosaic virus (TMV) RNA was isolated as described previously (8).

Protein Synthesis Assay. The methods used for the preparation of wheat germ extracts and conditions for protein synthesis were modified from Roberts and Paterson (8). The final assay contained 28 mM K *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.0, 3 mM magnesium acetate, 100 mM KCl, 40 μM spermine (free base), 1 mM dithiothreitol, 1 mM Tris ATP, 25 μM Na$_3$GTP, 8.8 mM Tris creatine phosphate, 5 μg/ml of creatine phosphokinase, 30 μM of unlabeled amino acids, [^{35}S]methionine (Amersham-

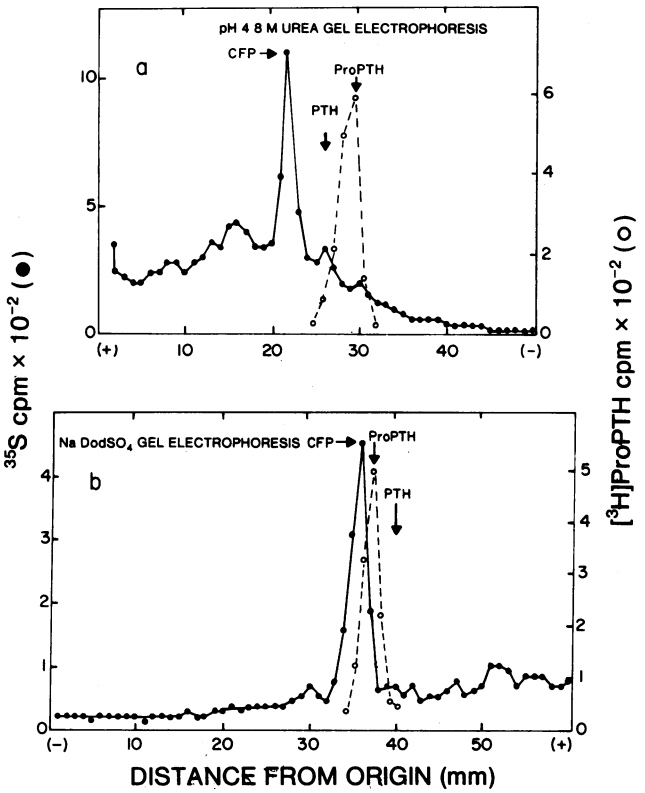

FIG. 1. Analysis by acrylamide gel electrophoresis of the cell-free products of protein synthesis directed by a 8–15S fraction of RNA from parathyroid glands. The proteins labeled with [^{35}S]methionine from a cell-free incubation were prepared for gel electrophoresis as described in *Methods*. [^{3}H]ProPTH, isolated as described previously (5), was added as a marker. The position of PTH relative to the cell-free product (CFP) was determined on separate gels. (a) The equivalent of 10 μl of a 25-μl incubation analyzed in 10% acrylamide gels at pH 4 in 8 M urea. (b) A protein sample corresponding to 2.5 μl of the incubation mixture was adjusted to 0.1 M sodium phosphate, pH 7.2, 0.5% Na $DodSO_4$, 4 M urea, and 1% 2-mercaptoethanol and heated to 100° for 30 sec before Na $DodSO_4$–acrylamide gel electrophoresis. ●, [^{35}S]methionine-labeled cell-free proteins; ○, [^{3}H]ProPTH.

Searle) or [^{3}H]lysine (New England Nuclear), and wheat germ extract (30,000 × *g* supernatant) to produce a final concentration of 5 mg/ml of protein determined by the Lowry method (9). Reactions of 25–100 μl were incubated at 23° for 3 hr. One-microliter aliquots of the incubation mixture, with 250 μg of carrier bovine serum albumin, were added to 1 ml of 10% trichloroacetic acid. The precipitate was pelleted by centrifugation, redissolved in 0.2 M NaOH, and reprecipitated with 10% Cl_3CCOOH. The precipitate was collected on Whatman GF/C glass fiber pads and radioactivity was analyzed by liquid scintillation counting (5). To further analyze the products of cell-free synthesis, 200 μg of a partially purified preparation of PTH (Cl_3CCOOH powder, Wilson Laboratories) was added as carrier to the reaction mixture and 10% Cl_3CCOOH was added. The precipitate was collected by centrifugation, dissolved in 0.2 M NaOH and reprecipitated with 10% Cl_3CCOOH. The precipitate was again centrifuged and then resuspended in water, lyophilized, and redissolved in 8 M urea–0.1 M acetic acid for polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described previously (5) except that Na $DodSO_4$ gels contained 4 M urea instead of 8 M urea.

TABLE 1. *Stimulation of protein synthesis in extracts of wheat germ by parathyroid RNA*

RNA added	Amino-acid incorporation (cpm)*	
	[^{35}S]Methionine	[^{3}H]Lysine
None	16,000	5,000
Parathyroid RNA	455,000	91,000
TMV RNA	2,920,000	590,000

Four micrograms of 8–15S parathyroid RNA and 1.3 μg of TMV RNA was incubated in 25-μl reaction mixtures for 3 hr at 23°. Ten microcuries of [^{3}H]lysine (33 Ci/mmole) or 5 μCi of [^{35}S]methionine (132 Ci/mmole) were added to the reaction.

* Total Cl_3CCOOH-insoluble radioactivity.

Isolation of PTH and ProPTH and the Product of Cell-Free Synthesis. Slices of parathyroid glands were incubated as described previously (5) at 37° with 100 μCi/ml of [methyl-^{3}H]-methionine (10.6 Ci/mmole, New England Nuclear) in Dulbecco's modified Eagle's medium minus methionine or with 12.5 μCi/ml of [^{14}C]lysine (0.3 Ci/mmole, New England Nuclear) in Earle's balanced salt solution containing 0.2 mM of the other 19 unlabeled amino acids. Protein was extracted from the tissue with 8 M urea–0.2 M HCl and prepared for polyacrylamide gel electrophoresis (5). PTH, ProPTH, and the major cell-free product were extracted after electrophoresis from 1-mm slices of Na $DodSO_4$–polyacrylamide gels with 0.2 ml of 0.05 M sodium phosphate, pH 7.2, 0.1% Na $DodSO_4$, 1 mM phenylmethylsulfonylfluoride. Small aliquots of the gel extract were assayed for radioactivity by scintillation counting and combined peak fractions of radioactivity were adjusted to 10% Cl_3CCOOH with 300 μg/ml of parathyroid Cl_3CCOOH powder (Wilson Laboratories) added as carrier. The precipitate was pelleted by centrifugation at 10,000 × *g* for 15 min and was extracted twice with acetone–ether (1:1) to remove Cl_3CCOOH and Na $DodSO_4$.

Protein Cleavage. The isolated [^{35}S]methionine-labeled cell-free product and [^{3}H]methionine-labeled PTH were resuspended together in 100 μl of 0.2 M ammonium bicarbonate (pH 8.5). About 250 μg of carrier Cl_3CCOOH powder was present. Five micrograms of trypsin was added at the beginning and after 1.5 hr during a total incubation at 37° of 3 hr. The peptides were analyzed by paper chromatography in butanol–acetic acid–water (4:1:5) as described (5).

For cyanogen bromide cleavage, isolated [^{3}H]lysine-labeled cell-free product was combined with either [^{14}C]-lysine-labeled PTH or ProPTH. About 80 μg of protein were dissolved in 50 μl of 70% formic acid, and 900 μg of cyanogen bromide was added at the beginning and after 5 hr of 15-hr incubations at 25°. The samples were lyophilized and analyzed by Na $DodSO_4$–acrylamide gel electrophoresis.

Immunoprecipitation. A 15-μl aliquot of the cell-free incubation mixture was added to 0.2 ml of 0.02 M sodium phosphate, pH 7.2, 5% fetal bovine serum. Aliquots of this sample were incubated with a guinea pig antiserum to bovine PTH (GP-1) or a control guinea pig serum, and the radioactivity bound to the antibodies was precipitated with a rabbit antiserum to guinea pig gamma globulin as described previously (5). The precipitated proteins were analyzed by Na $DodSO_4$–acrylamide gel electrophoresis.

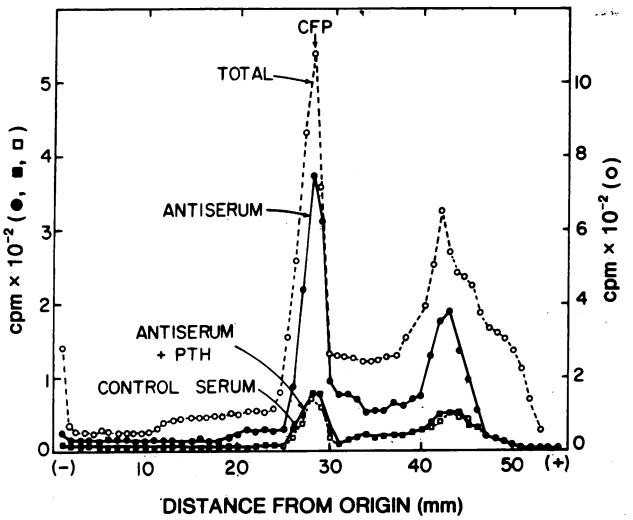

FIG. 2. Na $DodSO_4$-acrylamide gel analysis of immunoprecipitates of [^{35}S]methionine-labeled cell-free products. Part of complete incubation mixture (O); immunoprecipitate fractions from incubation mixture after treatment with antiserum to PTH (●); antiserum to PTH and 4 μg of PTH (■); nonimmune control serum (□). The results of the four acrylamide gels are coplotted in the figure. CFP refers to the major cell-free product.

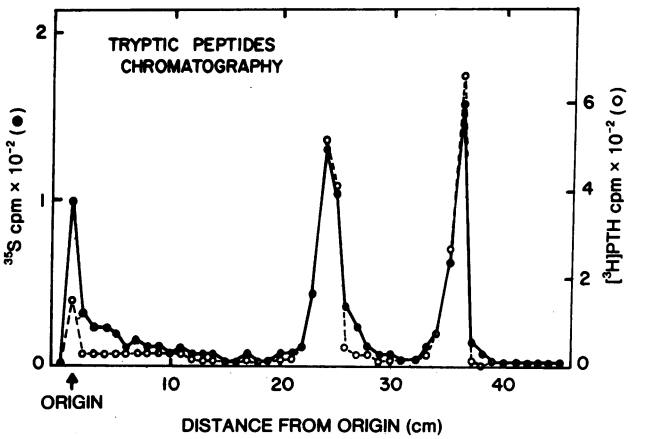

FIG. 3. Methionine-labeled tryptic peptides of the cell-free product and PTH. The major cell-free product labeled with [^{35}S]methionine and [^{3}H]methionine-labeled PTH were isolated from Na $DodSO_4$-acrylamide gels and digested with trypsin as described in *Methods*. The tryptic peptides were analyzed by paper chromatography on Whatman 3MM chromatography paper with butanol–acetic acid–water (4:1:5) (5). ●, [^{35}S]-methionine-labeled peptides of the cell-free product; O, [^{3}H]-methionine-labeled peptides of PTH.

RESULTS

Addition of parathyroid RNA isolated from bovine parathyroid glands to the wheat germ extract stimulated incorporation of either [^{3}H]lysine or [^{35}S]methionine by 15- and 30-fold, respectively (Table 1). The rate of incorporation was directly proportional to the amount of RNA added from 1 to 4 μg (the maximum amount added)/25 μl of incubation mixture. For comparison, TMV RNA (1.3 μg) was about six times as effective in stimulating protein synthesis as was the maximum amount of parathyroid RNA added (Table 1). Incorporation of radioactive amino acids into protein was linear for up to 3 hr for both TMV RNA and parathyroid RNA. Analysis of the radioactive products of cell-free synthesis by polyacrylamide gel electrophoresis at pH 4 resulted in one major radioactive fraction that migrated more slowly than either PTH or ProPTH (Fig. 1a). Na $DodSO_4$–acrylamide gel electrophoresis also revealed a single major component that migrated more slowly than either PTH or ProPTH (Fig. 1b). This suggests that the major cell-free product is both larger than ProPTH and more negatively charged. The amount of radioactivity in the major component on the Na $DodSO_4$–acrylamide gels represents about 25% of the total radioactivity incorporated into protein.

To determine whether the protein in the major peak might have structural components identical to PTH, the total reaction mixture was incubated with antiserum to PTH and the protein bound by the antiserum was precipitated and analyzed on Na $DodSO_4$-acrylamide gels (Fig. 2). A major radioactive component and a second component migrating more rapidly were observed. The proteins in both fractions bind to the antiserum and are precipitated. Addition of unlabeled PTH reduces the amounts of radioactivity bound in both fractions by about 80%. The small amount of radioactivity remaining in the fractions for which PTH does not compete is probably nonspecific binding, since a similar amount is bound by control serum that does not contain antibodies to PTH. These two proteins, thus, contain antigenic determinants in common with PTH. The protein in the second fraction may be a fragment of the larger protein in the major fraction: its relative amount appears to be a characteristic of different wheat germ extracts (in the experiment in Fig. 1, for example, this second fraction was not observed). The translation products of TMV RNA in the wheat germ system also contain some peptides smaller than expected (8, 10). These observations may be related to *in vitro* premature chain termination, as observed in extracts of Krebs II ascites cells (11, 12).

PTH contains two methionines, one in the amino-terminal tryptic peptide and the other in the tryptic peptide penultimate from the amino terminus (13, 14). The major product of the wheat germ extract also contained two methionine-containing tryptic peptides that had mobilities and stoichiometries identical to those of PTH when analyzed by paper chromatography (Fig. 3). This demonstrates that at least a portion of the cell-free product is similar to PTH. The fact that the cell-free product appears to be larger than ProPTH on Na $DodSO_4$-acrylamide gels indicates that it has additional amino acids. Three possible structures of this protein are illustrated at the top of Fig. 4. The extra amino acids could be present at the amino terminus (A) or at the carboxyl terminus (B and C). The additional hexapeptide at the amino terminus of ProPTH (15–17) could be removed by tryptic activity in the wheat germ extract, resulting in a cell-free product as in C. We have incubated ProPTH in wheat germ extracts for 3 hr under conditions normally used for protein synthesis and no conversion to PTH was observed. Thus, structure C is unlikely. Cyanogen bromide cleaves at the carboxyl side of the two methionines at positions 8 and 18 of PTH and produces two small peptides from the amino terminal end and one large peptide at the carboxyl end (Fig. 4). Since the cell-free product from the wheat germ extract does not contain any additional methionine residues (Fig. 3), cleavage of this protein will yield a large carboxyl terminal peptide identical to those of PTH and ProPTH if structure A in Fig. 4 is correct. If structure B or C is correct, the carboxyl terminal peptide of the cell-free product should migrate differently from those

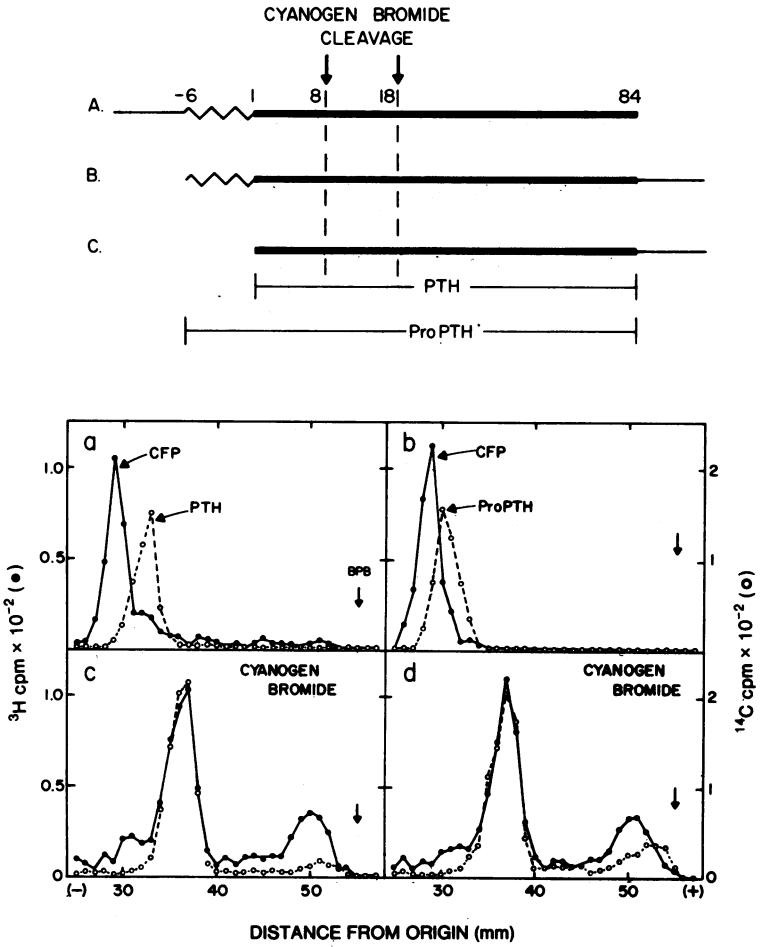

FIG. 4. Cyanogen bromide cleavage of the cell-free product, PTH, and ProPTH. The cell-free product was labeled with [^{3}H]lysine, and PTH and ProPTH were labeled with [^{14}C]lysine. The model at the top is a schematic representation of three possible structures of the wheat germ product. The heavy straight lines represent PTH, which contains 84 amino acids; the zigzag lines represent the extra hexapeptide present in ProPTH; and the light straight lines represent the extra amino acids present in the cell-free product. Cyanogen bromide cleaves at the carboxyl side of the two methionines at position 8 and 18. The large peptide 19–84 contains 8 lysines; 9–18 contains 1; 1–8 contains 0; and (−6)–8 contains 3. In panels a and b the analysis of the cell-free product with either PTH or ProPTH on Na DodSO$_4$–acrylamide gels is shown. In c and d analysis on Na DodSO$_4$–acrylamide gels is shown of samples (containing twice as much material initially as in a or b) after treatment with cyanogen bromide. CFP refers to the cell-free product. BPB and the arrows indicate the position of the bromphenol blue dye marker. ●, [^{3}H]lysine-labeled cell-free product; ○, [^{14}C]lysine-labeled PTH or ProPTH.

of PTH or ProPTH. Isolated [^{3}H]lysine-labeled cell-free product and [^{14}C]lysine-labeled PTH or ProPTH are clearly separated by electrophoresis on polyacrylamide gels in the presence of Na DodSO$_4$ (Fig. 4a and b). After treatment with cyanogen bromide the large carboxyl terminal peptide of the wheat germ product has a mobility identical to those of the corresponding peptides of PTH and ProPTH (Fig. 4c and d). This indicates that the cell-free product contains additional amino acids at the amino terminus when compared to ProPTH. Although the small amino terminal peptides of the cell-free product and ProPTH (Fig. 4d), observed near the bottom of the acrylamide gels (45–55 mm), are not well resolved, the amino terminal peptides of the cell-free product migrate more slowly than the corresponding peptides of ProPTH. This supports the conclusion that extra amino acids are present at the amino terminus of the cell-free product.

DISCUSSION

These studies demonstrate that RNA from calf parathyroid glands actively directs the synthesis of parathyroid proteins in a wheat germ extract. The major cell-free product has considerable structural identity to PTH, as evidenced by common antigenic sites and tryptic peptides, but is distinctly different from ProPTH or PTH, appearing to be more negatively charged than both ProPTH and PTH and slightly larger than ProPTH, as determined by gel electrophoretic analysis. Removal of small amino terminal peptides by cyanogen bromide produces a large carboxyl terminal peptide that is not detectably different for PTH, ProPTH, and cell-free product. This indicates that the cell-free product contains extra amino acids at the amino terminal end and not at the carboxyl terminus. On the basis of these data, we suggest that the cell-free product may be a precursor to PTH that is converted to ProPTH, which in turn is converted to PTH. We have named this protein pre-proparathyroid hormone (pre-ProPTH).

It is possible that pre-ProPTH is an artifactual product of the cell-free system, particularly since we have been unable to detect pre-ProPTH in intact parathyroid cells after a 2-min pulse with [^{3}H]leucine (unpublished observations). Four

basic types of errors are possible in the cell-free system: (*1*) missense errors, insertion of the wrong amino acids, (*2*) termination errors, (*3*) initiation errors, and (*4*) proteolytic cleavage of the product. The chromatographic identity of the methionine-containing tryptic peptides of PTH and pre-ProPTH indicates that no major missense errors occur in the wheat germ extract. These two peptides contain 13 of the 18 different amino acids present in PTH and 20% of the total amino acids. Since the carboxyl terminal peptides after cyanogen bromide treatment of pre-ProPTH and PTH have the same electrophoretic mobility on Na $DodSO_4$-acrylamide gels, the termination of the assembly of the protein appears to be executed reasonably accurately. Although the extra amino acids at the amino terminus could be the result of false initiation, this would require a second, specific, in-phase, initiator codon on the messenger RNA. Translation in the wheat germ extract of a second messenger RNA isolated from hyperplastic human parathyroid tissue produced a protein slightly larger than human ProPTH that is analogous to bovine pre-ProPTH (J. F. Habener, B. Kemper, J. T. Potts, Jr., and A. Rich, unpublished observations). This argues against a unique false initiation site in the bovine PTH messenger RNA. Finally, there is very low protease activity in the wheat germ extract, as measured in chymotryptic or tryptic assays (18). The contention that pre-ProPTH is not an artifactual translation product of the wheat germ cell-free system is also supported by the fact that a number of messenger RNAs coding for viral and eukaryotic proteins are faithfully translated in this system (8, 10, 19, 20). Thus, there is no compelling evidence, at present, to suggest that pre-ProPTH is not the direct product (minus the initiator methionine) of the translation of PTH messenger RNA.

We could not detect pre-ProPTH in intact cells after a short 2-min incubation (unpublished observations). A number of studies on the biosynthesis of PTH in bovine parathyroid slices have demonstrated the existence of a precursor to PTH that was designated ProPTH (or calcemic factor A) (5, 6, 21, 22). Pre-ProPTH is clearly different from this precursor and was not detected during short or long incubations with radioactive amino acids in several different media in these studies. Thus, pre-ProPTH may be converted rapidly to ProPTH *in vivo*, perhaps even before the completion of the synthesis of the carboxyl terminus. It seems possible that under unusual circumstances pre-ProPTH may not be cleaved and may accumulate in the cell or even be secreted. Several large proteins, immunoreactive to PTH antisera, have been found in hyperplastic parathyroid tissue (23) and in blood of patients with ectopic hyperparathyroidism (24). Pre-ProPTH may correspond to one of the immunoreactive species noted in these studies.

The properties of pre-ProPTH are similar to those of a precursor that has been described for the light chain of myeloma proteins (2). Both proteins are apparently cleaved rapidly and are not normally observed in the intact cell. Pre-ProPTH may function, as has been suggested for the pre-myeloma light chain, to facilitate the transport of the protein through the membrane or to initiate the binding of ribosomes translating new messenger RNA to the endoplasmic reticulum (2). Pre-ProPTH and the myeloma light chain precursor may represent a second class of protein precursors which have extremely short lives and may play a role early in the intracellular processing of secretory proteins. The proproteins (see ref. 1 for references) like ProPTH and proinsulin, which are relatively stable, may function in the later transport or secretion of the proteins.

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