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CELL-FREE TRANSLATION OF PROLINE-RICH PROTEIN mRNAS FROM HUMAN SUBMANDIBULAR GLAND

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SUMMARY: RNA was isolated from a human submandibular gland and separated into poly A-enriched and poly A-deficient fractions by chromatography on oligo(dT) cellulose. Both of these RNA fractions stimulated methionine incorporation into polypeptides in a reticulocyte lysate translation system. Two in vitro translation products templated by poly A-enriched mRNA were isolated by immunoprecipitation with immune serum directed against human salivary anionic proline-rich protein I. These polypeptides were shown to be precursors of proline-rich proteins on the basis of M_r, affinity for the antiserum, and preferential incorporation of proline. This study is the first to demonstrate cell-free translation of the mRNAs for human proline-rich salivary protein precursors.

The four major anionic proline-rich phospho-proteins (PRP I-IV) are principal components of human parotid and submandibular secretions accounting for 10-30% of the total protein (1,2). The primary structure of each of these secretory proteins has been determined (3-6). The two larger PRPs, PRP I and II, each contain 150 amino acid residues and have identical amino acid sequences except that residue four in PRP I is asparagine and residue four in PRP II is aspartic acid. The two smaller PRPs, PRP III and IV, each contain 106 residues with sequences identical to the amino terminal 106 residues of PRP I and II, respectively. The PRPs in human parotid and submandibular secretions display a genetic polymorphism showing three phenotypes characterized by the presence of the protein pair, PRP I and III, the protein pair PRP II and IV, or all four PRPs (7).

Functional studies have shown that the PRPs are potent inhibitors of seeded precipitation from solutions supersaturated with respect to calcium phosphate salts (8). Consequently, PRPs constitute an important oral defense

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system by providing thermodynamic driving forces unfavorable for dissolution of tooth enamel as well as unwanted precipitation of calcium phosphate salts in glandular ducts and on tooth surfaces.

We have shown immuno-histochemically at the light and electron microscopic level that serous acinar cells of both human and subhuman primate parotid and submandibular glands are the site of proline-rich protein synthesis (2,9). However, the molecular basis of the genetic polymorphism among human PRPs, or for that matter, the biosynthetic interrelationship among the four PRPs, is not known. The present work was initiated as a first step toward determining whether PRP I-IV are separate gene products or whether the larger PRPs I and II are proteolytically cleaved to the smaller PRPs, III and IV, respectively. We report here the isolation of translatable mRNA from a human submandibular gland and identification of PRP precursors among the translation products.

MATERIALS AND METHODS

Materials. A submandibular gland was obtained at autopsy from an 81 year old male 6 hours post-mortem. The gland, which showed no signs of pathology, was kept frozen in liquid nitrogen until used.

RNA Isolation. Frozen tissue (8.6 g) was fragmented into small pieces with mortar and pestle under liquid nitrogen and transferred to 20 ml of extraction buffer (0.02 M Tris-HCl, pH 8.0, containing 0.075 M NaCl, 0.025 M ethylenediamine tetraacetic acid, and 0.5% sodium dodecylsulfate) plus 10 ml of phenol saturated with extraction buffer, and homogenized with a polytron (Brinkmann Instruments, Westbury, NY). After the addition of a further 30 ml of extraction buffer and 40 ml of buffer saturated phenol, the homogenate was kept on ice for 30 min, centrifuged at 10,000 x g for 10 min, and the aqueous phase was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with chloroform:isoamyl alcohol (24:1, v/v). Nucleic acids in the resulting aqueous phase were recovered by ethanol precipitation overnight at -20°C and RNA was further purified by repeated precipitation in the presence of 3 M sodium acetate, pH 6.0, to remove DNA and low molecular weight RNAs (10). The partially purified RNA was ethanol precipitated and twice extracted with 2 M lithium chloride in 50 mM sodium acetate, pH 5.0, and ethanol precipitated again. Poly A-enriched and poly A-deficient RNA fractions (henceforth referred to as poly A^+ and poly A^- RNA, respectively) were prepared by oligo(dT) cellulose chromatography (Type II; Collaborative Research, Waltham, MA) (11).

Cell-Free Translation. RNA fractions were translated in a reticulocyte lysate as described by Pelham and Jackson (12). The standard translation reaction (25 ul) contained 10 ul nuclease treated lysate, 8 uM hemin, 12 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) buffer pH 7.6, 20 ug/ml creatine phosphokinase, 5 mM creatine phosphate, 110 mM potassium acetate, 0.25 mM magnesium chloride, 120 uM unlabeled amino acids minus the radioactive amino acid used, 160-320 ug/ml RNA, and 20 uCi of L-(^{35}S)-methionine (1200 Ci/mmol), L-(^{3}H)-proline (100 Ci/mmol) or

L-(^{3}H)-valine (57 Ci/mmol; all isotopes from Amersham, Arlington Heights, IL). Translation reactions were incubated for 35 min at 30°C.

Gel Electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed according to Laemmli (13). Gels were calibrated with both globular proteins and collagen derived cyanogen bromide peptides. Based on the electrophoretic mobility of globular protein standards, collagen-like proteins and PRPs due to their high proline content display erroneous M_r values (14). The M_r values given for native PRPs and translated PRP-precursors are those obtained by reference to collagen cyanogen bromide peptide standards.

Immunoprecipitation. Rabbit antiserum was obtained by immunization with PRP I and demonstrated equal avidity for all major anionic PRPs as described (15).

Cell-free translation products were isolated by immunoprecipitation as described by Foster et al. (16) except that antisera were added after incubating the translation reaction for 35 min and the 1X buffer contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 0.1% Nonidet P-40, 0.1% sodium deoxycholate and 2 mM methionine, proline or valine depending on which was used as the radiolabeled amino acid in the translation reaction. Heat-killed, formalin-fixed Staphylococcus aureus was obtained from The Enzyme Center, Boston, MA. Immunoprecipitates were centrifuged through a 1 M sucrose cushion in 1X buffer, taken up in 6 M urea, 62.5 mM Tris-HCl, pH 7.5, containing 2% sodium dodecylsulfate and 5% β-mercaptoethanol, and examined electrophoretically. Fluorography was performed with En^{3}Hance (New England Nuclear, Boston, MA) exposing dried gels to Kodak XAR-5 film at -80°C.

RESULTS

Human submandibular gland poly A$^+$ and poly A$^-$ mRNA stimulated incorporation of radiolabeled methionine into polypeptides in a reticulocyte lysate translation system (Table I). Poly A$^+$ mRNA promoted methionine incorporation into polypeptides (11-19 fold over lysate minus added RNA) to a

Table I. Translation of human submandibular mRNAs in a reticulocyte lysate system.

RNA Sample	Experiment 1		Experiment 2	
	ug RNA added	cpm/ug RNA/ 25 ul#	ug RNA added	cpm/ug RNA/ 25 ul#
Submandibular poly A$^+$ mRNA	2.0	503,000	4.0	128,000
Submandibular poly A$^-$ mRNA	8.0	42,000	8.0	33,000
Algal poly A$^+$ mRNA Standard*	-	-	8.0	229,000

#each 25 ul translation contained 20 uCi of L-(^{35}S)-methionine.
*The poly A$^+$ mRNA standard was obtained from the unicellular rhodophyte, Cyanidium caldarium (H. S. Belford, G. D. Offner, and R. F. Troxler, J. Biol. Chem. in press).

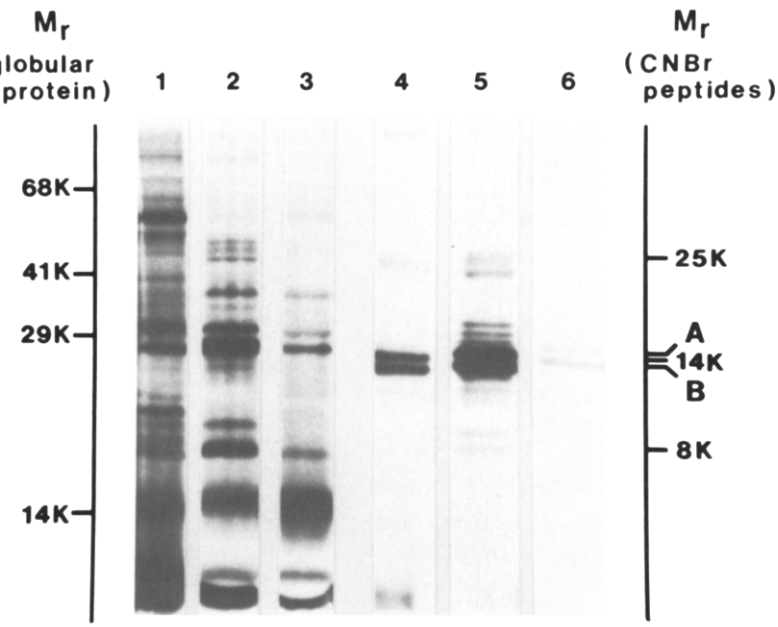

Figure 1. Analysis of polypeptides from poly A^+ mRNA directed translations by gel electrophoresis and fluorography. Lanes 1-3 show the profile of polypeptides labeled with methionine, proline and valine, respectively. Lanes 4-6 show the polypeptides isolated by immunoprecipitation from the translations depicted in lanes 1-3, respectively. Lanes 1-6 contained 160,000 cpm, 40,000 cpm, 34,000 cpm, 49,000 cpm, 50,000 cpm and 12,000 cpm. Fluorography of the dried gel was performed for 48 hours.
A, PRP-precursor A; B, PRP-precursor B; CNBr, cyanogen bromide.

much greater extent than poly A^- mRNA (6 fold over endogenous) presumably because the latter contained primarily ribosomal RNA. Gel analysis of acetone precipitable polypeptides showed that both poly A^+ and poly A^- mRNAs directed in vitro translation of more than 20 polypeptides ranging in M_r from approximately 12,000 to greater than 70,000 (data not given).

Translation reactions were carried out with poly A^+ mRNA template and with methionine, proline, or valine as the radiolabeled amino acids. Analysis of acetone precipitable polypeptides revealed numerous bands located throughout the entire length of the gel (Figure 1, lanes 1-3). One band, with an apparent M_r of 28,000 by reference to globular protein standards, represented one of the polypeptides labeled heavily with proline (Figure 1, lane 2). This band was labeled to a lesser extent with methionine and valine. We have shown M_r estimates of proline-rich proteins to be more realistic when gels are calibrated with proline-rich standards such as collagen cyanogen

Table II. Apparent M_r values for PRP I, PRP III, and the PRP-precursors A and B, isolated by immunoprecipitation with antiserum specific for PRPs.

Protein	Apparent M_r*	
	Collagen Cyanogen Bromide Peptide Standards	Globular Protein Standards
PRP I	12,500	26,000
PRP III	11,200	23,500
PRP-precursor A	14,600	29,000
PRP-precursor B	13,200	27,000

*Globular protein standards were bovine serum albumin, ovalbumin, alcohol dehydrognease, carbonic anhydrase, trypsin inhibitor and RNase A. Cyanogen bromide peptides were obtained from the α_1(I) chain of chick skin collagen (14). The apparent M_r values for PRPs in gels calibrated with collagen cyanogen bromide peptides are in good agreement with the M_rs of 15,627 for PRP I and 11,145 for PRP III, based on the amino acid sequence (3-6).

bromide peptides (14). The proline-rich translation product with an apparent M_r of 28,000 displayed an apparent M_r of 14,600 based on the electrophoretic mobility of collagen cyanogen bromide peptides.

Immunoprecipitates were prepared from the methionine, proline and valine translation reactions using immune serum directed against PRP I (Figure 1, lanes 4-6). Antiserum to PRP I, which cross-reacts with all four major anionic PRPs (15), exhibited a high degree of specificity for two translation products, designated as PRP-precursors A and B, with apparent M_rs of 14,600 and 13,200. These two translation products are presumed to represent precursors of PRPs on the basis of their cross-reactivity with the antiserum and preferential incorporation of radiolabeled proline. This is consistent with the amino acid composition of the PRPs where PRP I and II each contain 41 proline residues and PRP III and IV each contain 24 proline residues (3-6). Incorporation of radiolabeled valine into PRP-precursor A and B was significantly lower than incorporation of radiolabeled proline, as expected from the fact that each of the four PRPs contains only 3 valine residues (3-6). It is noteworthy that methionine was incorporated into the PRP-precursors A and B in view of the absence of methionine in mature PRPs (17).

The apparent M_rs of PRP-precursors A and B were between 2000 and 3000 daltons greater than those of PRP I and III, respectively, in gels calibrated either with globular proteins or collagen cyanogen bromide peptides (Table II). That the M_r values of the immunoprecipitated translation products are slightly larger than the native PRPs suggests that the former may contain a signal peptide such as those found at the amino terminus of most secretory proteins (18). The occurrence of a signal peptide in the in vitro synthesized precursors of PRPs would be predicted because the PRPs are secretory proteins (1,2,18). Moreover, this would explain methionine incorporation into these PRP precursors because eukaryotic protein synthesis is initiated with methionine (19) and one or more methionine residues have been found in most signal peptides for which the amino acid sequence has been determined (20).

DISCUSSION

Azen and Oppenheim (7) showed by gel analysis of saliva samples from 240 human subjects that PRPs display genetic polymorphism reflected by three phenotypes. Analysis of the data are consistent with a polymorphism controlled by two codominant autosomal alleles where the allele Pr^1 codes for PRP I and III and the allele Pr^2 codes for PRP II and IV. It was not clear from these results whether the Pr^1 locus contains separate genes for PRP I and III, or whether PRP III arises from PRP I by post-translational proteolysis. The same questions apply to the Pr^2 locus and the relationship between PRP II and IV. Since PRP I and III, and PRP II and IV occur in parotid and submandibular secretions in a ratio approaching 1:1, it seems unlikely that the 1:1 stoichiometry of the respective PRP pairs could be explained by a proteolytic processing event. Nevertheless, there is no information available as to whether the four major PRPs are, in fact, the products of four distinct genes.

The PRP-precursors A and B templated in vitro with human submandibular poly A^+ mRNA (a) cross-reacted with an immune serum specific for PRPs, (b) were proline-rich, (c) contained methionine which is absent in the mature PRPs, and (d) displayed apparent M_r values 2000 daltons greater than native PRP I and III. This strongly suggests that these in vitro translation products

are precursors of the PRPs and have a methionine-containing signal peptide. In addition, it is highly likely that the PRP-precursors A and B arise from distinct mRNAs, which is strong evidence that the larger PRPs I and II are not cleaved to the smaller PRPs III and IV, but rather, that all four PRPs arise from four different structural genes. We cannot determine as yet whether PRP-precursor A repesents PRP I, or PRP II, or both, because these PRPs have virtually the same M_r and it is not known whether the individual from which the submandibular gland was obtained was homozygous or heterozygous for the Pr^1 and Pr^2 alleles. Similarly, the present results do not allow PRP-precursor B to be ascribed to the presence of PRP III, or PRP IV, or both. The identity of PRP-precursors A and B can be readily determined using recombinant DNA technology. We have already made cDNA from the human submandibular poly A^+ mRNA (S. P. Rogelj, S. R. Farmer, H. S. Belford, R. F. Troxler and F. G. Oppenheim, unpublished results). Gel analysis of this cDNA revealed two major bands with 500 and 700 base pairs. These bands may correspond to the mRNA sequences for the larger PRPs, I and II, and the smaller PRPs, III and IV, based on their abundance, size, and the fact that they are cytosine rich.

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REFERENCES

1. Baum, B. J., Kousvelari, E. E., and Oppenheim, F. G. (1982) J. Gerontol. 37, 392-395.
2. Kousvelari, E. E., Baratz, R. S., Burke, B. and Oppenheim, F. G. (1980) J. Dent. Res. 59, 1430-1438.
3. Wong, R. S. C., Hofmann, T. and Bennick, A. (1979) J. Biol. Chem. 254, 4800-4808.
4. Wong, R. S. C. and Bennick, A. (1980) J. Biol. Chem. 255, 5943-5948.
5. Schlesinger, D. H. and Hay, D. I. (1979) Peptides. Proc. 6th American Peptide Symposium (Gross, E. and Meienhofer, J. eds) pp. 133-136. Pierce Chem. Co., Rockford, IL.
6. Schlesinger, D. H. and Hay, D. I. Personal Communication.
7. Azen, E. A. and Oppenheim, F. G. (1973) Science 180, 1067-1069.
8. Hay, D. I., Moreno, E. C., and Schlesinger, D. H. (1979) Inorg. Perspec. Biol. & Med. 2, 271-285.
9. Kousvelari, E. E., Oppenheim, F. G. and Cutler, L. S. (1982) J. Histochem. & Cytochem. 30, 274-278.
10. Kirby, K. S. (1965) Biochem. J. 96, 266-269.
11. Aviv, H. and Leder, P. (1972) Proc. Nat. Acad. Sci. (US) 69, 1408-1412.
12. Pelham, H. R. B. and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247-256.

13. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685.
14. Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (1982) J. Biol. Chem. 257, 9271-9282.
15. Kousvelari, E. E. and Oppenheim, F. G. (1979) Biochim. Biophys. Acta 578, 76-86.
16. Foster, J. A., Rich, C. B., Fletcher, S., Karr, S.R. and Przybzla, A. (1980) Biochemistry 19, 857-864.
17. Oppenheim, F. G., Hay, D. I. and Franzblau, C. (1971) Biochemistry 10, 4233-4238.
18. Sabbatini, D. D., Kreibich, G., Morimoto, T. and Adesnik, M. (1982) J. Cell Biol. 92, 1-22.
19. Maitra, U., Stringer, E. A. and Chaudhuri, A. (1982) Ann. Rev. Biochem. 51, 869-900.
20. Kreil, C. (1981) Ann. Rev. Biochem. 50, 317-348.