Biosynthesis of Salivary Proteins in the Parotid Gland of the Subhuman Primate, *Macaca fascicularis*

CELL-FREE TRANSLATION OF THE mRNA FOR A PROLINE-RICH GLYCOPROTEIN AND PARTIAL AMINO ACID SEQUENCE AND PROCESSING OF ITS SIGNAL PEPTIDE

(Received for publication, July 18, 1983)

Heather S. Belford, Elisa G. Triffleman, Gwyneth D. Offner, Robert F. Troxler, and Frank G. Oppenheim

From the Department of Biochemistry, Boston University School of Medicine, and Department of Oral Biology, Goldman School of Graduate Dentistry, Boston, Massachusetts 02118

The major anionic proline-rich proteins in the parotid and submandibular secretions of subhuman primates and man perform the important biological function of inhibiting crystal growth of calcium phosphate salts from saliva, which is supersaturated with calcium phosphate salts, thereby preventing excess deposition of hydroxylapatite on tooth surfaces. The present work was initiated as a first step towards investigating proline-rich protein biosynthesis in parotid glands using the subhuman primate, *Macaca fascicularis*, as a model system. RNA was isolated from macaque parotid glands and separated into poly(A)-enriched and poly(A)-deficient fractions by chromatography on oligo(dT)-cellulose. The mRNAs in both fractions promoted incorporation of radiolabeled amino acids into polypeptides in an mRNA-dependent reticulocyte lysate translation system. Five major proline-rich polypeptides were detected and one of these was shown to be the in vitro precursor of the major anionic macaque proline-rich protein (MPRP), which is the structural and functional counterpart of the major anionic proline-rich proteins in the parotid and submandibular secretions of man (Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (1982) J. Biol. Chem. 257, 9271–9282). Radiosequencing of the material in anti-MPRP immune precipitates showed that the in vitro precursor of MPRP contained an 18-residue signal peptide. The in vitro precursor of MPRP was processed in dog pancreas vesicles to a form with a lower apparent Mr, and with an NH₂-terminal amino acid sequence identical to that of native MPRP. The phenylthiohydantoin derivatives of Ala and Ile were detected at residue 9 and those of Val and Met were detected at residue 16 of the signal peptide. This indicated that the in vitro precursor of MPRP, which migrated electrophoretically as a single band in anti-MPRP immune precipitates, contained two different in vitro polypeptides derived from two different mRNAs. These results are discussed in the context of the genetic polymorphism among the major anionic proline-rich proteins in the parotid and submandibular secretions of man.

The major anionic proline-rich proteins constitute up to 30% of the protein in the parotid and submandibular secretions of man (1). The PRPs comprise a family of homologous proteins that display a genetic polymorphism with 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 (2). These proteins, together with the tyrosine-rich phosphopeptide, statherin (3), inhibit spontaneous precipitation of calcium phosphate salts and crystal growth of calcium phosphate salts in vitro (4). Since saliva is supersaturated with calcium phosphate salts, the biological function of the major anionic PRPs appears to be to maintain saliva supersaturated with respect to calcium phosphate and to prevent excess deposition of calcium phosphate on tooth surfaces (4, 5).

Little is known about the molecular events occurring during the biosynthesis of PRPs. Meunzer et al. (6, 7) reported that chronic treatment of rats with the β-agonist, isoproterenol, resulted in hypertrophy of the parotid glands and concomitantly, expression of the genes for six basic proline-rich proteins. The six basic proline-rich proteins were the primary translation products templated by poly(A⁺) mRNA from the parotid glands of isoproterenol-treated animals, whereas these proteins were essentially undetectable in translation reactions templated by poly(A⁺) mRNA from untreated animals (8). While the isoproterenol-treated rat parotid gland is an interesting system for investigating the effects of catecholamines on gene expression, the biological function of basic proline-rich proteins in the oral cavity of rats and man is not known.

We described the cell-free translation of the mRNAs for precursors of PRPs' from a human submandibular gland and identified these precursors on the basis of cross-reactivity with immune serum specific for PRPs and preferential incorporation of radiolabeled proline (9). The disadvantages of using human parotid or submandibular glands reside in the limited availability of glandular tissue and the complexity of the system due to the genetic polymorphism among the PRPs (2).

We have isolated a proline-rich glycoprotein (MPRP) which is a major component in the parotid and submandibular secretion of the subhuman primate, *Macaca fascicularis* (10). The chromatographic and electrophoretic properties and amino acid composition of MPRP are very similar to those of...
the PRPs. MPRP and the PRPs display a 68% homology within the NH2-terminal 66 residues (10, 11). Kousaveli et al. (12, 13) have shown immunohistochemically at the light and electron microscopic level the anomalous acinar cells of both human and macaque parotid and submandibular glands, lending support to the site of prolactin-rich protein biosynthesis. MPRP and the PRPs have comparable activities in the crystal growth inhibition assay (10), leaving little doubt that MPRP is the macaque counterpart of the major anionic PRPs of man. Further, M. fascicularis is known to develop a number of human diseases under experimental conditions including periodontitis and caries (14).

In the present investigation, isolation of translatable mRNA from the macaque parotid gland and characterization of the in vitro precursor of MPRP are described.

**EXPERIMENTAL PROCEDURES**

**Materials**—Parotid glands were surgically removed from adult animals within 1 h of sacrifice, frozen in liquid nitrogen, and stored at −80 °C until used.

**RNA Isolation**—Frozen tissue (approximately 8 g) was broken into small pieces with a mortar and pestle under liquid nitrogen and transferred to 20 ml of extraction buffer consisting of 0.02 M Tris-HCl, pH 8.0, 0.075 M NaCl, 0.025 M EDTA, and 0.5% sodium dodecyl sulfate. Subsequently, 10 ml of phenol saturated with extraction buffer was added and the tissue was homogenized in a Polytron (Brinkmann Instruments, Westbury, NY). After the addition of a further 30 ml of extraction buffer and 40 ml of phenol saturated with extraction buffer, the homogenate was kept on ice for 30 min, and centrifuged at 10,000 × g for 10 min. The aqueous phase was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). Nucleic acids in the resulting aqueous phase were recovered by ethanol precipitation overnight at −20 °C. The RNA was further purified by repeated precipitation in the presence of 3 M sodium acetate, pH 6.0, to remove undegraded 18 and 28 S ribosomal RNAs.

RNA was chromatographed on oligo(dT)-cellulose (Type II; Collaborative Research, Waltham, MA) as described by Aviv and Leder (16). The poly(A)-enriched and poly(A)-deficient fractions (subsequently referred to as poly(A)+ and poly(A)− RNAs, respectively) were ethanol precipitated and stored at −80 °C. The poly(A)+ fraction was used for making agarose gels (17) and shown to contain undegraded 18 and 28 S ribosomal RNAs.

In one experiment, the poly(A)+ fraction was chromatographed on poly(U) agarose (P-L Biochemicals). The poly(A)+ fraction was applied to the column in binding buffer composed of 50 mM Tris-HCl, pH 7.5, 0.7 M NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 25% formamide (v/v). The poly(A)+ fraction (not retained on the column in binding buffer) was recovered by the addition of 2 volumes of ethanol and stored at −20 °C.

**Cell-free Translation**—Reticulocyte lysates were prepared from rabbits made anemic with acetylsalicylhydroxide and lysates were stored under liquid nitrogen. Prior to use, lysates were made mRNA-dependent by limited digestion with micrococal nuclease (Boehringer Mannheim) as described by Pěham and Jackson (18). The standard 20-μl translation reaction contained: 10 μl of nuclease-treated lysate, 12 mM HEPES buffer, pH 7.6, 5 μM bovin, 20 μg/ml of creatine phosphokinase, 5 mM creatine phosphate, 110 mM potassium acetate, 0.25 mM magnesium chloride, 120 μl unlabelled amino acids minus the radiolabeled amino acid used, 1-10 μg of RNA, and 1 μCi/μl of [35S]methionine (Amersham Corp. 1200-1500 Ci/mmol) or one of the following titrated amino acids: L-[3H]leucine, L-[3H]isoleucine, L-[3H]valine, L-[3H]phenylalanine, or L-[3H]proline (Amersham Corp. 10-130 Ci/mmol).

Translation reactions were incubated for 35 min at 30 °C and incorporation of radiolabeled amino acids into high trichloroacetic acid-insoluble material was determined (19). For analysis, 15 volumes of ice-cold acetone containing 2% concentrated HCl (v/v) were added to 1 volume of translation reaction and the resulting precipitate was washed with ice-cold acetone. The washed precipitates were taken up in 62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol (v/v), 2% sodium dodecyl sulfate, and 5% β-mercaptoethanol (sample buffer), heated at 100 °C for 2 min, and examined electrophoretically.

**Gal Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (19) on 12.5% gels in which the ratio of acrylamide to N,N':N,N'-methylenebisacrylamide monomer was 1:18.5. Fluorography was performed by exposure of the dried gel to Kodak XAR-5 film at −80 °C.

**Immunoprecipitation**—Preparation and specificity of rabbit antisera directed against MPRP and PRP I have been described previously (18). PRP I immune serum cross-reacts with native PRPs I, II, III, and IV.

Isolation of translation products from reticulocyte lysates by immune precipitation was performed as described by Belford et al. (21).

Briefly, 1 volume of 250 mM Tris-HCl, pH 7.5, containing 750 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 10 μl unlabelled amino acid (5 × buffer) was added to 4 volumes of translation reaction. The appropriate immune serum, (2 μl/100-μl reaction volume) was added and the mixture was allowed to stand overnight at 4 °C. Subsequently, 1 volume of 30% (v/v) heat-killed, formalin-fixed Staphylococcus aureus (The Enzyme Center, Boston, MA) was added to 2 volumes of immunoprecipitation mixture, and homogenized on a temperature controlled microsonicator (M-1772). Spermidine-phosphat ed Edman degradation (22) was carried out on a Beckman 890C Sequencer equipped with a cold trap using program 121078 with 0.25 μM Quadrol and a combined S1 and S2 wash. The background of each radiosequencing run was reduced by performing a wash step (no coupling reagent) followed by double coupling. Phenylthiohydantoin norleucine was added to each fraction collector tube to serve as an internal standard.

The butyl chloride in fraction collector tubes was evaporated to dryness under nitrogen. The residues were dissolved in 1.0 ml of butyl chloride and 0.5 ml was assayed for radioactivity in a liquid scintillation spectrometer. The remaining 0.5 ml of butyl chloride was evaporated to dryness and the amino acids were hydrolyzed in 6 N HCl. The amino acid phase was dried under a stream of nitrogen. The residue was taken up in 40 μl of methanol and analyzed on a Waters high pressure liquid chromatography apparatus equipped with a C8 column as described (24). The eluate from the C8 column was collected in 1.0-ml fractions and each fraction was assayed for radioactivity.

The PTH derivatives of radiolabeled amino acids were identified by comparing the elution volume of radioactivity with that of unlabeled PTH amino acid standards. Repetitive yields were determined from the recovery of the PTH derivatives of valine (steps 1 and 10) and leucine (steps 2, 9, and 11) of the sperm whale apomyoglobin standard.

**Processing of the in Vitro Precursor of MPRP in Dog Pancreas Vesicles**—Macaque parotid gland poly(A)+ mRNA was translated in an mRNA-dependent reticulocyte lysate obtained commercially (Amersham Corp. Catalog No. N-90). The translation reactions contained: 14 μl of reticulocyte lysate, 2 μg of mRNA 1.2 μg of dog pancreas vesicles (New England Nuclear; 1:4 dilution of vesicles as supplied), and 1 μCi/μl of L-[3H]proline, L-[3H]methionine, or L-[3H]valine (Amersham Corp.). Translation reactions were incubated for 35 min at 30 °C, made 2% with respect to sodium dodecyl sulfate and heated to 100 °C for 2 min. After cooling on an ice bath, 4 volumes of 1.0 × immunoprecipitation buffer and 2 μl of anti-MPRP immune serum were added and the mixtures were kept at 4 °C overnight. Heat-killed, formalin-fixed, S. aureus was added and the solid material was recovered by centrifugation through 1 M sucrose as described above. Translation products were eluted from the washed pellet with sample buffer for electrophoretic analysis or with 70% formic acid for radiosequencing.

For gel analysis, 15 volumes of ice-cold acetone containing 2% concentrated HCl (v/v) were added to 1 volume of translation reaction and the resulting precipitate was washed with ice-cold acetone. The washed precipitates were taken up in 62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol (v/v), 2% sodium dodecyl sulfate, and 5% β-mercaptoethanol (sample buffer), heated at 100 °C for 2 min, and examined electrophoretically.
RESULTS

Cell-free Translation of the in Vitro Precursor of MPRP—

Macaque parotid poly(A+) mRNA and poly(A−) mRNA promoted incorporation of L-[3H]proline, L-[35S]methionine, and L-[3H]isoleucine into acetone-insoluble polypeptides in an mRNA-dependent reticulocyte lysate (Table I). The stimulation of radiolabeled amino acid incorporation was approximately 5 times greater per μg of RNA with poly(A+) mRNA template. Examination of the translation products showed that the profile of radiolabeled polypeptides was essentially the same with either poly(A+) mRNA or poly(A−) mRNA templates (Fig. 1). Separate rechromatography of the poly(A+) mRNA on oligo(dT)-cellulose, or of the poly(A−) mRNA on poly(U) agarose, did not alter the profile of polypeptides templated by either fraction or decrease template activity (counts/min incorporated/μg of RNA).

L-[3H]Proline was preferentially incorporated into 5 polypeptides, designated bands I–V (Fig. 1), which represent the major proline-rich polypeptides whose mRNAs were extracted from the parotid gland and translated in vitro. We show below that the material in band V is the in vitro precursor of native MPRP. MPRP is the single, major proline-rich protein in macaque parotid secretion functionally equivalent to the human PRPs (10). We have been unable to establish which of the native proline-rich proteins in macaque parotid secretion correspond to the in vitro polypeptides contained in bands I–IV. However, it is certain that these polypeptides (bands I–IV) are not the in vitro forms of macaque parotid gland proteins analogous to PRPs I–IV in human parotid secretion because MPRP is the only functionally equivalent proline-rich protein in the macaque (10).

The apparent Mr values for proline-rich proteins are erroneously high in gel filtration and electrophoretic systems calibrated with globular protein standards. Somewhat more accurate Mr values are obtained when such systems are calibrated with proline-rich polypeptide standards (10, 26). The apparent Mr values of MPRP, PRPs, and the polypeptides in bands I–V, on slab gels calibrated with both globular protein standards and chick skin collagen α1(I) chain CNBr peptides, are given in Table II. These data show that the apparent Mr values of these proteins are about 1.5 times greater when estimated by reference to the calibration curve for globular protein standards versus collagen CNBr peptides. The apparent Mr values of native MPRP, PRPs, and the polypeptides in bands I–V, on slab gels calibrated with both globular protein standards and chick skin collagen α1(I) chain CNBr peptides, are given in Table II. These data show that the apparent Mr values of these proteins are about 1.5 times greater when estimated by reference to the calibration curve for globular protein standards versus collagen CNBr peptides. The apparent Mr values of native MPRP, PRPs, and the polypeptides in bands I–V, on slab gels calibrated with both globular protein standards and chick skin collagen α1(I) chain CNBr peptides, are given in Table II. These data show that the apparent Mr values of these proteins are about 1.5 times greater when estimated by reference to the calibration curve for globular protein standards versus collagen CNBr peptides. The apparent Mr values of native MPRP, PRPs, and the polypeptides in bands I–V, on slab gels calibrated with both globular protein standards and chick skin collagen α1(I) chain CNBr peptides, are given in Table II. These data show that the apparent Mr values of these proteins are about 1.5 times greater when estimated by reference to the calibration curve for globular protein standards versus collagen CNBr peptides.

Immune precipitates prepared with anti-MPRP immune serum from the poly(A+) mRNA directed translation reactions (Fig. 1, lanes 1–3) contained one major component with an apparent Mr approximately 5 times greater when estimated with the collagen CNBr peptide calibration curve. These values are in close agreement with the Mr values calculated from the amino acid composition or amino acid sequences of these proteins (10, 27–29). For this reason, the Mr values of translation products subsequently referred to are those obtained from gels calibrated with collagen CNBr peptides.

TABLE I

<table>
<thead>
<tr>
<th>Isotope</th>
<th>RNA Fraction</th>
<th>Amount</th>
<th>Incorporation ratio: poly(A+) / poly(A−)</th>
<th>Radioactivity* (cpm/μg RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[3H]Proline</td>
<td>Poly(A+)</td>
<td>1.6</td>
<td>58,000</td>
<td>1.6</td>
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<tr>
<td></td>
<td>Poly(A−)</td>
<td>7.0</td>
<td>45,000</td>
<td>7.0</td>
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<tr>
<td>L-[3H]Isoleucine</td>
<td>Poly(A+)</td>
<td>1.6</td>
<td>43,000</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Poly(A−)</td>
<td>7.0</td>
<td>8,000</td>
<td>7.0</td>
</tr>
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</table>

* Values represent counts/min/μg of RNA incorporated into hot trichloroacetic acid-insoluble material in a 25-μl translation reaction.

FIG. 1. Analysis of translation products from macaque parotid gland poly(A+) mRNA and poly(A−) mRNA directed translation reactions by gel electrophoresis and fluorography. Lanes 1, 2, and 3 show acetone-insoluble polypeptides from a poly(A+) mRNA directed translation reaction containing L-[3H]proline, L-[35S]methionine, or L-[3H]isoleucine, respectively. Lanes 4, 5, and 6 show acetone-insoluble polypeptides from poly(A−) mRNA directed translation reactions containing radiolabeled proline, methionine, and isoleucine, respectively. 35,000 cpm was added to each lane. Globular protein standards: bovine serum albumin, (68,000); ovalbumin (43,000); alcohol dehydrogenase (41,000); carbonic anhydrase (29,000); RNase A (13,700). Collagen cyanogen bromide peptides from chick skin collagen α1(I) chain: CB8 (24,800); CB3 (13,800); CB6A (8,570); CB6B2 (7,910). Major proline-rich translation products are designated bands I–V.
TABLE II
Apparent Mₐ values of MPRP, PRPs, and proline-rich polypeptides translated in a reticulocyte lysate containing macaque poly(A⁺) (or poly(A⁻)) mRNA

Electrophoresis was performed on 10% slab gels (19) calibrated with globular protein standards and collagen cyanogen bromide peptides. The proline-rich polypeptides in bands I–V refer to those shown in Fig. 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Apparent Mₐ (kDa)</th>
<th>Globular protein standards*</th>
<th>Collagen cyanogen bromide peptide standards*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPRP</td>
<td>28,600 ± 400</td>
<td>19,800 ± 100</td>
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</tr>
<tr>
<td>PRP I or II</td>
<td>22,000 ± 500</td>
<td>14,300 ± 500</td>
<td></td>
</tr>
<tr>
<td>PRP III or IV</td>
<td>19,500 ± 400</td>
<td>12,200 ± 500</td>
<td></td>
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<tr>
<td>Band I</td>
<td>33,800 ± 300</td>
<td>22,800 ± 1000</td>
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</tr>
<tr>
<td>Band II</td>
<td>26,200 ± 700</td>
<td>17,500 ± 900</td>
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<tr>
<td>Band III</td>
<td>23,000 ± 800</td>
<td>14,500 ± 800</td>
<td></td>
</tr>
<tr>
<td>Band IV</td>
<td>20,000 ± 600</td>
<td>12,400 ± 400</td>
<td></td>
</tr>
<tr>
<td>Band V</td>
<td>19,700 ± 300</td>
<td>11,500 ± 100</td>
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</table>

* Native MPRP is a phosphoglycoprotein in which the minimum Mₐ of the polypeptide moiety is 13,240 and the minimum Mₐ of the carbohydrate moiety is 4,150 (10, 11). The Mₐ values of PRP I and III calculated from the amino acid sequence are 15,627 and 11,145, respectively (27, 28).

**The Mₐ values of globular protein standards and collagen CNBr standards are given in the legend to Fig. 1.

Fig. 2. Analysis of anti-MPRP immune precipitates from poly(A⁺) mRNA directed translation reactions by gel electrophoresis and fluorography. Lane 1, acetone-insoluble polypeptides from a translation reaction containing L-[³⁵S]methionine (53,000 cpm); lane 2, anti-MPRP immune precipitate from the translation reaction shown in lane 1 (8000 cpm); lane 3, anti-MPRP immune precipitate from a translation reaction containing L-[³⁵S]methionine (8000 cpm); lane 4, anti-MPRP immune precipitate from a translation reaction containing L-[³⁵S]methionine (8000 cpm).

apparent Mₐ of 11,500 (Fig. 2). This component displayed an electrophoretic mobility identical to that of the polypeptide in band V. The anti-MPRP immune serum showed a weak affinity for the polypeptides in bands I–IV, but these components were quantitatively insignificant in the immune precipitates. The polypeptide in band V was the least abundant proline-rich translation product (Fig. 1, lanes 1 and 4) but the main component in the anti-MPRP immune precipitate, and on this basis was designated the in vitro precursor of MPRP. Anti-MPRP immune precipitates from translation reactions templated by poly(A⁻) mRNA containing radiolabeled proline, methionine, or isoleucine were identical to those in Fig. 2 (data not given).

Automated Edman Degradation of the in Vitro Precursor of MPRP—Radiolabeled methionine was incorporated into the in vitro precursor of MPRP, but native MPRP does not contain methionine (30). This suggested that a methionine-containing signal peptide occurred at the NH₂ terminus of the in vitro precursor of MPRP.

Macaque parotid gland poly(A⁺) mRNA was translated in reticulocyte lysates containing L-[³⁵S]methionine, L-[³⁵S]valine, L-[³⁵S]isoleucine, L-[³⁵S]alanine, or L-[³⁵S]leucine and the material in anti-MPRP immune precipitates from these translation reactions was subjected to automated Edman degradation.

Radiosequencing of the material in the anti-MPRP immune precipitate from the translation reaction containing radiolabeled methionine showed that the PTH derivative of methionine occurred at steps 1, 13, and 16 (Fig. 3A). This is consistent with the premise that methionine occurred at residues 1, 13, and 16 of a signal peptide at the NH₂ terminus of the in vitro precursor of MPRP.

Radiosequencing of the in vitro precursor of MPRP in the immune precipitate from the translation reaction containing radiolabeled valine revealed that the PTH derivative of valine was present at steps 8, 16, 23, and 28 (Fig. 3B). Valine is located at residues 5 and 10 of native MPRP (10, 11). Therefore, it was concluded that the PTH derivative of valine at steps 23 and 28 of the in vitro precursor of MPRP corresponded to valine at residues 5 and 10 of the native protein. If correct, this would mean that the in vitro form of MPRP contains an 18-residue signal peptide.

Radiosequencing of isoleucine- or alanine-labeled material in immune precipitates demonstrated that the PTH derivative of isoleucine was located at steps 4, 9, and 17, and that the PTH derivative of alanine occurred at steps 9 and 12 (Fig. 3C and D). The radioactivity observed at step 1 in the analysis of isoleucine-labeled material was an artifact because no radiolabeled PTH-isoleucine was detected when the sample was examined by high pressure liquid chromatography. The PTH derivative of leucine was detected at steps 2, 3, 5, 6, 10, and 11 by radiosequence analysis of immune precipitate from a translation reaction carried out with radiolabeled leucine (Fig. 3E). From the foregoing results, a partial amino acid sequence of the signal peptide at the NH₂ terminus of the in vitro precursor of MPRP was deduced (Scheme 1).

Both methionine and valine occurred at residue 16 and both alanine and isoleucine occurred at residue 9 of the MPRP signal peptide. This can be explained if two different MPRP precursors, derived from two different mRNAs, were isolated from translation reactions with anti-MPRP immune serum. The observed microheterogeneity precluded unambiguous determination of the complete amino acid sequences of either signal peptide by radiosequencing techniques, and further experiments to detect further microheterogeneity were not performed.

Processing of the in Vitro Precursor of MPRP—The supposition that the in vitro precursor of MPRP contains an 18-residue signal peptide is based on assignment of PTH-[³⁵S]valine at steps 23 and 28 to residues 5 and 10 of the native protein. This was confirmed by showing that dog pancreas vesicles processed the in vitro MPRP precursor to a polypeptide with a smaller Mₐ which lacked methionine and had an
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Fig. 3. Radiosequence analysis of the in vitro precursor of MPRP isolated by immune precipitation from poly(A*) mRNA directed translation reactions containing L-[35S]methionine (A), L-[3H]valine (B), L-[3H]isoleucine (C), L-[3H]alanine (D), or L-[3H]leucine (E). The partial amino acid sequence of the signal peptide at the NH2 terminus of the in vitro precursor of MPRP deduced from these results is shown in Scheme 1. The repetitive yields of the sperm whale aponymoglobin standard in A–E ranged from 92–97%. In A, 250,000 cpm were applied to the Sequencer cup; in B, 50,000 cpm; in C, 77,000 cpm; in D, 50,000 cpm; in E, 60,000 cpm. The radioactivity of the respective PTH derivatives in Sequencer cycles was determined as described under “Experimental Procedures.” Even though a preliminary wash step was performed on all samples, significant radioactivity (200–500 cpm) was noted in the first Sequencer cycle in most experiments with tritiated amino acids. Subsequent analysis by high pressure liquid chromatography showed that this radioactivity did not co-elute with the PTH derivative of the tritiated amino acid used in that experiment.

NH2-terminal sequence corresponding to that of native MPRP.

Macaque parotid gland poly(A*) mRNA was translated in reticulocyte lysate with radiolabeled methionine, or valine, with or without dog pancreas vesicles. In the translation reaction containing radiolabeled proline and dog pancreas vesicles, processing of all proline-rich polypeptides (bands I–V) was noted (Fig. 4, lanes 1 and 2). The apparent M, values of these polypeptides decreased from 200 to 1100 daltons when compared to proline-rich polypeptides from control translations without dog pancreas vesicles (Table III). Processing of the in vitro precursor of MPRP (band V) resulted in a decrease in the apparent M, from 11,500 to 10,500 (Fig. 4, lanes 3 and 4). In this experiment, processing of the MPRP precursor was about 80% complete as indicated by the presence of the M, = 10,500 and 11,500 bands in a ratio of approximately 4:1 (Fig. 4, lane 4). The extent to which the in vitro form of MPRP was processed by dog pancreas vesicles in the experiments depicted in Figs. 4 and 5 was 80 and 50%, respectively. The explanation for this result is not clear.

With the commercial reticulocyte lysate (Amersham Corp.) the profile of proline-rich proteins was the same as that seen using the lysate prepared according to the procedure of Pelham and Jackson (18). However, the in vitro precursor of MPRP was a much more prominent band in the commercial lysate even though the poly(A*) template was the same used with the other lysate (compare Fig. 1, lane 1 and Fig. 4, lanes 1 and 2). We cannot explain this observation.

Macaque parotid gland poly(A*) mRNA was translated in reticulocyte lysates containing radiolabeled methionine, with or without dog pancreas vesicles. In the presence of dog pancreas vesicles, the polypeptides in bands I–V were difficult to detect whereas these polypeptides were present in translation reactions minus dog pancreas vesicles (data not given) and identical to those in Fig. 1 (lane 2). Furthermore, band V was not detected in the anti-MPRP immune precipitate from the translation reaction containing dog pancreas vesicles (data not given). These results provide indirect evidence that each proline-rich polypeptide (bands I–V) contains a signal peptide with one or more methionine residues and that the processed polypeptides lack this amino acid.

Macaque parotid gland poly(A*) mRNA was next translated in a reaction containing L-[3H]valine and dog pancreas vesicles. Electrophoretic analysis of the anti-MPRP immune precipitate demonstrated that the in vitro precursor of MPRP had been partially processed due to the presence of both M, = 10,500 and 11,500 bands in the immune precipitate. Automated Edman degradation of the material in the immune precipitate showed that the PTH derivative of valine occurred at steps 5, 8, 10, and 16 (Fig. 5). The interpretations of these data were that the PTH-[3H]valine at steps 5 and 10 corresponded to residues 5 and 10 of the in vitro MPRP precursor from which the signal peptide had been cleaved and that the PTH-[3H]valine at steps 8 and 16 corresponded to valine at residues 8 and 16 of the signal peptide.

Comparative Immunology—Macaque parotid gland poly(A*) mRNA and poly(A*) mRNA were translated in a reticulocyte lysate with L-[3H]proline as the radiolabeled amino acid. Immune precipitates were prepared using antisera directed against either MPRP or against the human proline-rich protein, PRP I. As noted above, anti-MPRP immune precipitates contained primarily the in vitro precursor of MPRP (band V: apparent M, = 11,500) (Fig. 6, lanes 2 and 4). However, anti-PRP I immune precipitates did not contain the in vitro precursor of MPRP but instead contained the proline-rich polypeptides corresponding to bands II, III, and IV with a small amount of band I polypeptide (Fig. 6, lanes 1 and 3). The reason for failure of anti-PRP I immune serum to cross-react with the in vitro precursor of MPRP, even though this anti-serum cross-reacts with native MPRP, is difficult to explain. Nevertheless, the strong cross-reactivity between anti-PRP I immune serum and the macaque proline-rich polypeptides in Bands II, III, and IV is interesting and must reflect common antigenic determinants in PRP I and the macaque proline-rich polypeptides.

DISCUSSION

The present investigation is the first to demonstrate cell-free translation of the mRNAs for proline-rich proteins from the parotid gland of the subhuman primate, M. fascicularis. One of the proline-rich translation products (band V) was shown to be the in vitro precursor of MPRP on the basis of its apparent M, cross-reactivity with anti-MPRP immune serum, automated Edman degradation of the material in anti-
Translation of Macaque Parotid Gland mRNAs

IN VITRO

<table>
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<tr>
<th>Step</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>Met-Leu-Leu-Ile-Leu-Leu-Val-Ala-Leu-Ala-Met-</td>
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<td>X -</td>
<td>X -</td>
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<tr>
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<td>5</td>
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NATIVE PROTEIN: Pse-Pse-Glu-Asp-Val-Pse-Gln-Glu-Asp-Val-

Scheme 1. Alignment of the partial amino acid sequence of the in vitro form of MPRP with the NH2-terminal amino acid sequence of the native protein. Pse, phosphoserine.

Table III

<table>
<thead>
<tr>
<th>Band</th>
<th>Apparent M*</th>
<th>Translation reaction minus dog pancreas vesicles</th>
<th>Translation reaction plus dog pancreas vesicles</th>
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<tr>
<td>I</td>
<td>23,100</td>
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<tr>
<td>II</td>
<td>16,600</td>
<td>15,600</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>13,700</td>
<td>13,500</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>11,500</td>
<td>12,000</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>11,500</td>
<td>10,500</td>
<td></td>
</tr>
</tbody>
</table>

MPRP immune precipitates, and processing in dog pancreas vesicles to a polypeptide with an NH2-terminal amino acid sequence comparable to that of native MPRP. The in vitro precursor of MPRP contains a signal peptide at the NH2 terminus as has been reported for numerous other secretory proteins (31). Microheterogeneity in the signal peptide of the MPRP precursor suggested that band V contained two chemically distinct translation products derived from different mRNAs in the macaque parotid gland.

Several findings in the present work deserve further comment. First, the profile of proline-rich polypeptides templated by poly(A+) mRNA and poly(A-) mRNA was nearly identical (Fig. 1). The poly(A+) mRNA fraction displayed about one-fifth as much template activity as did the poly(A-) mRNA (Table I), yet examination of the poly(A-) fraction on denaturing agarose gels showed this fraction contained predominantly ribosomal RNA. Since most of the RNA after poly(A) selection was recovered in the poly(A-) fraction, and template activity for proline-rich polypeptides in the poly(A-) fraction was about one-fifth that of the poly(A+) mRNA fraction, a significant portion of the message population for proline-rich polypeptides did not bind to oligo(dT)-cellulose. Chromatography of the poly(A-) fraction on poly(U) agarose, a resin known to have a greater affinity for short poly(A) sequences (32), did not alter the template activity, indicating that some mRNAs for proline-rich polypeptides contain very short poly(A) sequences, or none at all. It is possible that removal of poly(A) sequences from the 3' region of these mRNAs occurs during processing (33) or that these mRNAs are not polyadenylated initially. The present work may be among the first in which mRNAs for secretory proteins have been found in both the poly(A+) mRNA and poly(A-) mRNA fractions. Parenthetically, the genes for PRPs in man are located on chromosome 1 (34). Thus, it would be expected that the genes for macaque parotid gland proline-rich proteins are also encoded in nuclear DNA and that the mRNAs derived from these genes would at some point become polyadenylated.

Second, we identified 5 major proline-rich polypeptides (bands I–V) among the translation products templated by mRNAs from the macaque parotid gland, and the polypeptide(s) in band V was unmistakably characterized as the in vitro precursor(s) of MPRP. The identity of the in vitro polypeptides in bands I–IV is not known, although these could be the macaque counterparts of the basic proline-rich proteins (35) and the basic proline-rich glycoprotein (36) in the parotid and submandibular secretion of man.

Fig. 4. Cell-free translation of macaque parotid gland poly(A+) mRNA in a reticulocyte lysate in the presence and absence of dog pancreas vesicles and with L-[3H]proline as the radiolabeled amino acid. The fluorogram shows acetone-insoluble polypeptides from translation reactions lacking (lane 1) and containing (lane 2) dog pancreas vesicles. Lanes 3 and 4 show the anti-MPRP immune precipitates from the translation reactions depicted in lanes 1 and 2, respectively. Radioactivity applied was as follows: lane 1, 275,000 cpm; lane 2, 290,000 cpm; lane 3, 19,000 cpm; lane 4, 18,000 cpm.
precipitates from a poly(A+) mRNA containing dog pancreas vesicles and L-[3H]valine. The amino acid sequences of the processed and unprocessed polypeptides are shown in the upper panel. Each Sequencer cycle was assayed as described under "Experimental Procedures" and the results are given in the lower panel. The PTH derivative of valine detected at steps 5 and 10 corresponds to valine at residues 5 and 10 of processed MPRP polypeptide; the PTH derivative of valine at steps 8 and 16 corresponds to valine at residues 8 and 16 of the signal peptide in the unprocessed precursor of MPRP. Approximately 20,000 cpm were applied to the Sequencer cup. The repetitive yield of the sperm whale apomyoglobin standard was 90%.

Third, radiosequence analysis of the material in anti-MPRP immune precipitates showed that the 18-residue signal peptide at the NH₂ terminus of the in vitro precursor of MPRP had alanine and isoleucine at residue 9, and methionine and valine at residue 16 (Fig. 3 and Scheme 1). The methionine/valine microheterogeneity can be explained by a 1-base change in the codon (third position), but the alanine/isoleucine microheterogeneity would require a change in the first 2 bases of the codons for the latter two amino acids. We have determined approximately 75% of the primary structure of native MPRP and have found no evidence for microheterogeneity in the amino acid sequence (10, 11). This suggests the possibility that the base sequence in the mRNAs for the two in vitro precursors of MPRP may be identical in the regions coding for the native protein whereas the base sequences coding for the signal peptides are clearly different. A somewhat similar situation has been described for mouse liver and salivary gland α-amylase mRNAs which have identical base sequences in the coding and nontranslated 3' region but different base sequences in the 5' untranslated regions (37, 38). Microheterogeneity in the amino acid sequences of the signal peptides of other secretory proteins such as rat proinsulin (39) and canine pretrypsinogen (40) has been described.

Finally, electrophoretic analysis of the material in anti-MPRP immune precipitates from reticulocyte lysates (Fig. 2) and automated Edman degradation of native MPRP (10, 11) indicate the presence of a single component both in the translation reactions and in the protein found in the parotid secretion of M. fascicularis, respectively. It is interesting, therefore, that radiosequencing experiments (Fig. 3) showed that the in vitro precursor of MPRP comprises two polypeptides. This suggests the possibility that there may be two genes for MPRP in M. fascicularis, as might be predicted considering the genetic polymorphism among human PRFs.

REFERENCES
Translation of Macaque Parotid Gland mRNAs

Biosynthesis of salivary proteins in the parotid gland of the subhuman primate, Macaca fascicularis. Cell-free translation of the mRNA for a proline-rich glycoprotein and partial amino acid sequence and processing of its signal peptide.

H S Belford, E G Triffleman, G D Offner, R F Troxler and F G Oppenheim