Cell-free Translations of Proline-rich Protein mRNAs*

Marilyn A. Ziemer, April Mason, and Don M. Carlson

From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

Treatment of rats with the $\beta$-agonist isoproterenol causes a dramatic increase in a series of proteins rich in proline (proline-rich proteins) in the porcine glands (Muenzer, J., Bildstein, C., Gleason, M., and Carlson, D. M. (1979) J. Biol. Chem. 254, 5623, 5629). These proteins which contain about 43% proline, comprise more than 50% of the total soluble protein of glands of rats treated with isoproterenol for 10 days. Further studies by in vitro translation analysis using the reticulocyte lysate system and labeling with $[^{3}H]$proline or $[^{35}S]$methionine show definitive changes in patterns of protein synthesis and proline-rich protein mRNAs are highly elevated in treated animals. Analysis of translation products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed (1) very little synthesis of proline-rich proteins from poly(A)$^+$ RNA of glands of normal rats, (2) poly(A)$^+$ RNA from glands of treated animals synthesize mainly proline-rich proteins, (3) translations with $[^{3}H]$proline and $[^{35}S]$methionine give identical labeling patterns of proline-rich proteins, and (4) proline-rich proteins from cell-free translations are all precipitated by antibodies to proline-rich proteins. At least six different proline-rich proteins are translated with poly(A)$^+$ mRNA from glands of treated animals. Each of these proteins is likely a translation product of a separate, specific mRNA. The dramatic changes in protein synthesis of rat parotid glands in response to isoproterenol treatment suggest that the para gland of the isoproterenol-treated rat is an excellent model system to study the overall responsiveness of gene expression to catecholamines.

Regulation of tissue-specific gene expression is poorly understood. Numerous enzymes and proteins of specific functional significance either for development, such as the silk moth chorion (1, 2), or for later function such as $\alpha$-amylase (3, 4) and ovalbumin (5, 6) have been studied in molecular regulation of tissue-specific gene expression is poorly understood. Numerous enzymes and proteins of specific functional significance either for development, such as the silk moth chorion (1, 2), or for later function such as $\alpha$-amylase (3, 4) and ovalbumin (5, 6) have been studied in molecular detail. The parotid glands of rats offer an interesting model system for studying the overall effects of catecholamines on gene expression. Profound morphological effects on rat salivary glands by adrenergic agonists, specifically isoproterenol, were reported in 1961 by Selye et al. (7) and by Brown-Grant (8). Repeated pharmacological doses caused glandular hypertrophy and hyperplasia. Biochemical changes resulting from isoproterenol treatment have been described, and especially a dramatic increase in a series of proteins high in proline, or PRP, by Muenzer et al. (9, 10). The high proline content of these proteins (43% Pro) and the dramatic increase in amounts, with PRPs comprising greater than 50% of the total glandular soluble protein in isoproterenol-treated rats, prompted us to study the synthesis of these unusual proteins. In this paper we report on the cell-free translation studies and demonstrate that each protein of a series of at least six proline-rich proteins is likely translated from a separate mRNA.

EXPERIMENTAL PROCEDURES

Materials and Methods—All materials were of highest purity available and were purchased from commercial sources unless otherwise indicated. The following substances were purchased from the respective companies: oligo(dT)-cellulose, Collaborative Research Inc.; micrococcal nuclease, Boehringer Mannheim; I$^{-}$-[3]$^{4}$H$^{+}$proline (40 to 60 Ci/mmole), and I$^{-}$-[3]$^{5}$S$^{2}$methionine (600 to 1300 Ci/mmole), Amerham Radio Chemicals; KB$^{-}$[3$^{3}$H]$^{+}$ (9.8 Ci/mmole), New England Nuclear. Rabbit antibody prepared against $\alpha$-amylase was a generous gift from Dr. W. J. Rutter, University of California, San Francisco. Radioactivity was measured by liquid scintillation counting. Aqueous samples were dissolved in 4 ml of Aquasol II (New England Nuclear) and filter papers were suspended in 4 ml of Beckman ReadySolv NA.

Isoproterenol Treatment—Rats were treated with isoproterenol-HCl as described previously (9). Treatment was for 10 days except where otherwise indicated.

Antiserum Preparation—Proline-rich proteins were isolated as described previously (9, 10). Basic proteins (IPR-1B, through 1B6) from CM-cellulose chromatography were combined and are referred to as “total B-PRP.” Rabbits were injected subcutaneous in four places on the back (3 mg of total B-PRP in 1 ml of sterile 140 mM NaCl plus 1 ml of complete Freund's adjuvant). Booster injections of 3 mg of total B-PRP in incomplete adjuvant were administered similarly every week for 2 months. Subsequently, boosters of 1 mg of total B-PRP were given every other week. Peak titer was reached after 2 months.

Tritium Labeling of PRP In Vitro—Tritium labeling of PRP was performed by reductive methylation as described by Kumarasamy and Symons (11). Products were purified by Sephadex G-55 chromatography. Total B-PRP, Ipr-1B2, Ipr-1B3, and Ipr-1B4 were labeled to specific activities (counts per min per microgram) of 3.6 x 10$^{3}$, 2.4 x 10$^{3}$, 3.0 x 10$^{3}$, and 1.8 x 10$^{3}$, respectively.

Antibody Precipitations—Samples were diluted to 0.1 ml in 2% Triton X-100 and 2% sodium deoxycholate. Ten ml of a solution of 20% serum in 2% Triton-DOC, and where indicated 10 to 25 $\mu$g competing antigen, were added and the mixture incubated at room temperature for 1.5 h. Goat anti-rabbit IgG (0.1 ml, prepared according to directions of supplier, Calbiochem-Behring) was added and the incubation was continued for 1 h. This reaction mixture was layered over a discontinuous gradient of 0.2 ml of 0.5 mM sucrose and 0.2 ml of 1.0 mM sucrose and centrifuged at 12,000 x g for 4 min. The pellets were washed four times by vigorous shaking in 1.0 mM sucrose and then dissolved in 0.5 ml of 10% acetic acid for liquid scintillation counting or in 30 ml of sample buffer (12) for SDS-polyacrylamide gel electrophoresis.

* This research was supported in part by Public Health Service Grants AM19175 and GM07076. This is Journal Paper 8966 from the Purdue University Agricultural Experiment Station. The costs of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

† Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

‡ Present address: Department of Nutrition, Purdue University, West Lafayette, IN 47907.

1 The abbreviations used are: PRP, proline-rich proteins; SDS, sodium dodecyl sulfate; Ipr used as a prefix, such as Ipr-1B, indicates specific protein fractions isolated from isoproterenol-treated rats (9).
Isolation of RNA—Total RNA was isolated essentially by the procedure reported by Harding et al. (13) using guanidine thiocyanate and mercaptoethanol, except that the pellet obtained after centrifugation through CsCl was redissolved in the homogenization buffer and then RNA was precipitated with ethanol. This pellet was washed in 70% ethanol and dissolved in water.

Oligo(dT)-cellulose chromatography of RNA was performed as described by Aviv and Leder (14). The polyadenylated RNA fraction was repeatedly passed over the column until retention was greater than 80%. RNA finally retained by the column is referred to as poly(A+) RNA.

In Vitro Translation Analysis—Reticulocyte lysate was prepared according to Darnbrough et al. (15) from rabbits injected with ace\n
tylphenylhydrazine. mRNA was translated in vitro as described by Bonner and Jackson (16) using the reticulocyte lysate. Translation incubations in 60 μl contained 37.5 μl of nuclease-treated lysate, 100 mM KCl, 0.5 mM MgCl2, 10 mM creatine phosphate, 25 μM hemin, 1 mM ATP, 0.2 mM GTP, 10 μg/ml of creatine phosphokinase, and unless otherwise stated, 40 μCi of radioiodinated amino acid, either [3H]proline or [35S]methionine, and 20 μg of total RNA or 5 μg of poly(A+) RNA. Reaction mixtures were incubated 90 min at 30 °C. Aliquots of the incubation mixtures (5 μl) were added to 1 ml of H2O, 0.5 ml of 10 mM NaOH/0.5 M H2O2 was added and incubated for 15 min at 37 °C to decolorize the hemin. One ml of cold 2% trichloroacetic acid/2.5% phosphotungstic acid was added and the precipitate was harvested on glass fiber filter discs (Whatman 934-AH, 2.4-cm diameter). Filters were washed successively with 10% trichloroacetic acid/1% perchloric acid. Filters were added to 0.5 ml of 10% trichloroacetic acid, dried, and counted. The addition of phosphotungstic acid is necessary because PRP are soluble in 15% trichloroacetic acid.

Products of in vitro translations were prepared for analysis by SDS-polyacrylamide gel electrophoresis by moving an aliquot, usually 5-μl, and by terminating the reaction by adding a solution of ribonuclease A (0.1 volume of 1 mg/ml in 50 mM sodium acetate, pH 5.0) and incubating for 15 min at 37 °C. The RNase A preparation was boiled for 5 min to denature proteins and DNases before it was added. Polypeptides in the nuclease-treated samples were precipitated with 8 volumes of acetone for 10 min in a dry ice bath or overnight at -20 °C. Precipitate was collected by centrifuging at 12,000 × g for 4 min, dried in vacuo and dissolved in 100 μl of sample buffer (12) with boiling for 3 min.

SDS-polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gels (13%, 30, 0.8 acrylamide, bisacrylamide) with 4.75% stacking gels (13%, 30, 0.8 acrylamide, bisacrylamide) were run at 30 mA per gel until the bromphenol blue dye was 1 cm from the bottom of the gel. Gels were prepared for fluorography as described by Bonner and Laskey (17). Dried gels were exposed to Kodak X-OMAT XRP film at -80 °C.

Treatment of Parotid Gland Slices—Two to 1 cm2 pieces of parotid gland (6 to 7 mg each) were incubated in 0.2 ml of Earle's balanced salt solution containing 33 pCi of either [3H]methionine or [35S]methionine, and then RNA was precipitated with ethanol. This pellet was washed with ethanol and dissolved in water.

RESULTS AND DISCUSSION

Cell-free Translation—The procedure developed by Harding et al. (13) for isolating RNA from cultured embryonic pancreas yielded intact, biologically active RNA from parotid glands of both normal and Ipr-treated rats. A second extraction with guanidine thiocyanate and mercaptoethanol was necessary to remove traces of ribonuclease. Protein synthesis measured by translation of total RNA from Ipr-treated rats was linear for 1 h. Incorporation of [35S]methionine was proportional to RNA concentration up to 10 μg of total RNA and 0.5 μg poly(A) RNA. The high proline content of PRP allowed for a comparison of labeling of proteins by [3H]proline and by [35S]methionine. For example, the incorporations of [3H]proline and of [35S]methionine using RNA from parotid glands of normal and Ipr-treated rats are shown in Table I. [3H]proline incorporation shows a 4-fold increase in translation products with RNA prepared from Ipr-treated rats, while [35S]methionine incorporation is essentially unchanged. In similar experiments, which compared the incorporation of [3H]leucine and [3H]proline, the difference in the proline to [35S]methionine ratios of translation products for RNAs from normal and Ipr-treated glands was about 8-fold (data not presented). This increase in proline incorporation is consistent with the in vivo incorporation of [3H]proline; greater than 97% of proline incorporated into protein by parotid glands of Ipr-treated rats was in PRP (9, 10). The data presented in Table I are definitive only if the same proteins, or in this case if proteins with similar proline and methionine compositions, are synthesized. However, native proline-rich proteins do not contain methionine and the proline-rich proteins labeled with [35S]methionine in cell-free translation experiments have the 35S-label only in the NH2-terminus. The methionine to proline ratio of the cell-free translation proline-rich proteins, calculated from the amino acid compositions and using a molecular weight of 17,000 (10), is about 1.54. Calculation of methionine and proline concentrations in the translation products using specific activities of the two isotopes and assuming little or no contribution from endogenous amino acids gives concentrations of 8 pmoles [35S]methionine and 390 pmoles [3H]proline, or a ratio of 1.50. It is important to recognize that almost all of the 35S-label and the H-label of translation products from mRNAs of Ipr-treated animals are in proline-rich proteins (Figs. 1 and 2).

Products of in vitro translations were prepared for analysis by SDS-polyacrylamide gel electrophoresis by moving an aliquot, usually 5-μl, and by terminating the reaction by adding a solution of ribonuclease A (0.1 volume of 1 mg/ml in 50 mM sodium acetate, pH 5.0) and incubating for 15 min at 37 °C. The RNase A preparation was boiled for 5 min to denature proteins and DNases before it was added. Polypeptides in the nuclease-treated samples were precipitated with 8 volumes of acetone for 10 min in a dry ice bath or overnight at -20 °C. Precipitate was collected by centrifuging at 12,000 × g for 4 min, dried in vacuo and dissolved in 100 μl of sample buffer (12) with boiling for 3 min.

SDS-polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gels (13%, 30, 0.8 acrylamide, bisacrylamide) with 4.75% stacking gels (13%, 30, 0.8 acrylamide, bisacrylamide) were run at 30 mA per gel until the bromphenol blue dye was 1 cm from the bottom of the gel. Gels were prepared for fluorography as described by Bonner and Laskey (17). Dried gels were exposed to Kodak X-OMAT XRP film at -80 °C.

Effect of Isoproterenol on Rat Parotid Glands

| RNA source                  | Amino acid incorporated | Relative abundance (%) | CPM × 10^-3/µg RNA |
|-----------------------------|-------------------------|------------------------|---------------------|
| [3H]Proline                 | [35S]Methionine         | 0.33                   |
| Normal                      | 36                      | 110                    |
| Isoproterenol-treated       | 150                     | 122                    |

1 Recent unpublished results by Dr. Haile Mehana of our laboratory show that the [35S]methionine incorporated during in vitro translation experiments is removed during the first cycle of peptide sequencing.

Effects of Isoproterenol on Rat Parotid Glands

Total RNA from parotid glands of normal and isoproterenol-treated rats was translated in standard reactions except that [3H]proline was present at 80 μCi/90 μl and incubations contained 10 μg RNA/60 μl. Values are averages of duplicate determinations.

| Product                        | Amino acid incorporated | Relative abundance (%) | CPM × 10^-3/µg RNA |
|--------------------------------|-------------------------|------------------------|---------------------|
| [3H]Proline and [35S]Methionine|                         |                        |                     |
| Normal                         | 36                      | 110                    | 0.33                |
| Isoproterenol-treated          | 150                     | 122                    | 1.23                |
accurate determination of relative mRNA concentrations will require analysis with hybridization probes for the various PRPmRNAs.

**Immunoprecipitation of Translation Products—The in vitrol**

![Image](http://www.jbc.org/Downloaded from)

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of translation products of RNA from rat glands treated for various times with isoproterenol. Products from translation of RNA with [35S]methionine were electrophoresed on 13% polyacrylamide gels. Each lane contains 46,000 cpm. RNA was obtained from rat glands treated with isoproterenol for zero (lane 1), 4(2), 6(3), 8(4), and 10(5) days.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of cell-free translation products immunoprecipitated with antibodies to proline-rich proteins and to a-amylase. Translation products of RNA obtained from rat glands of normal rat and of isoproterenol-treated rat were incubated with antiserum to proline-rich proteins (AP) and a-amylase (AA) and with pre-immune serum (NS). The precipitates were electrophoresed on 13% polyacrylamide gels and labeled proteins were detected by fluorography. Lanes 1 to 16 and lane 21 contain 23,000 cpm [35S]methionine-labeled translation products; lanes 17-20 contain 80,000 cpm [3H]proline-labeled translation products. NTP and ITP refer to translation products of RNA from normal and isoproterenol treated rats, respectively. Lanes 1 and 21, ITP; 12, NTP; and 17, ITP, are not treated with antiserum. Lanes 4 to 10 were immunoprecipitated in the presence of 15 µg of proline-rich proteins. Translation products of the various lanes were treated as follows: 2IAP, 3IAP, 4IAP, 5IAP, total B-PRP, 6IAP, PRP-1B; 7IAP, PRP-1B; 8IAP, PRP-1B; 9IAP, PRP-1B; 10IAP, PRP-1B; 11IAP, AA; 12NAP, AA; 14NAP, 15NAP, total B-PRP; 16NAP; 17IAP, 18IAP, 19IAP, 20IAP, total B-PRP.
erably higher specific activity. Small “slices” of parotid gland from isoproterenol-treated rats were incubated in short-term culture with [\(^{3}H\)]proline and [\(^{35}S\)]methionine (Fig. 3). Initial studies showed that optimal incorporation was obtained by a 2.5-h culture time and that the patterns of labeled proteins did not change between 0.5 and 3.5 h. Lanes 1 and 2 (Fig. 3) show proteins that were labeled in culture by [\(^{3}H\)]proline and [\(^{35}S\)]methionine, respectively. The \(H\)-labeled substances in lane 1, presumed to be PRP, could not be identified by immunoprecipitation. The relatively large amounts of endogenous PRP in the parotid gland slices inhibited immunoprecipitation of the labeled products because of the low antibody titer. Comparisons of lanes 1, 2, and 3 clearly show that PRP synthesized by gland slices are not labeled with [\(^{35}S\)]methionine. This lack of methionine is consistent with the amino acid compositions of PRP (10), and shows that methionine incorporated during translation is removed by post-translational processing. Additional post-translational processing may occur by removal of a \(NH\)\(_{2}\)-terminal amino acid sequence, or a signal peptide (18), as is common with most secreted proteins. The excision of a signal peptide is generally demonstrated by observing a difference in molecular weights of the mature protein and the larger cell-free translation product (19). In Fig. 3, it appears that proline-rich proteins 2, 3, and 4 (lane 3) may be precursors of proteins 2', 3', and 4' (lane 1). However, this association is as yet inconclusive.

The comparison of proteins synthesized by a cell-free mRNA-dependent translation system with mRNA from parotid glands of normal and isoproterenol-treated rats shows a dramatic change in gene expression and suggests that the responsiveness of rat salivary glands to \(\beta\)-adrenergic agonists offers an attractive model system for studying the overall effects of catecholamines on regulation of transcription. The in vitro studies reported earlier (9, 10) and the cell-free translation results reported here clearly show that proline-rich proteins comprise the major portion of proteins synthesized by parotid glands of isoproterenol treated rats. Both total RNA and poly(A\(^+\)) RNA were translated in the presence of [\(^{35}S\)]methionine and [\(^{3}H\)]proline. The same labeling patterns from each isotope were observed with RNAs from isoproterenol-treated animals when immunoprecipitated with PRP-antibodies and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography, even though the native proteins do not contain methionine. Sequence analysis was used to demonstrate that [\(^{35}S\)]methionine is present only on the \(NH\)\(_{2}\)-terminus.

An anomaly is observed when apparent molecular weights demonstrated by SDS-polyacrylamide gel electrophoresis are compared with molecular weights determined by sedimentation equilibrium studies and gel filtration (10). Sedimentation equilibrium studies showed molecular weights of 15,000 to 18,000, or essentially identical molecular weights for three of the proline-rich proteins (10). All proline-rich proteins eluted in the same position at an apparent molecular weight of 71,000 from Sephadex G-100 (9, 10). The Stokes radii of the proline-rich proteins obtained from gel filtration data were in good agreement with the calculated Stokes radii of 32 to 40 Å. Molecular weights observed by SDS-polyacrylamide gel electrophoresis vary from about 22,000 to 36,000 (Fig. 2). We have no explanation for these differences except that like collagen peptides (20), PRP migrate anomalously on SDS-polyacrylamide gels.

Results of this study suggest that separate and specific mRNAs are translated for each proline-rich protein since [\(^{35}S\)]methionine is incorporated into the \(NH\)\(_{2}\)-terminus of each protein by cell-free translation. Proteins synthesized in vitro do not contain methionine. These data are inconsistent with the hypothesis that the processing of larger peptides by proteolysis to form the family of proline-rich proteins.

Fig. 3. SDS-polyacrylamide gel electrophoresis of [\(^{3}H\)]proline and [\(^{35}S\)]methionine labeled products of parotid gland slice culture. Soluble fractions of cultured parotid gland slices were electrophoresed on 15% polyacrylamide gel and labeled proteins were detected by fluorography. Lane 1, 10 \(\mu\)l from [\(^{3}H\)]proline-labeled culture; lane 2, 10 \(\mu\)l from [\(^{35}S\)]methionine-labeled culture; lane 3, 38,000 cpn [\(^{35}S\)]methionine-labeled translation products (same as lanes 1 and 21, Fig. 2).
REFERENCES

1. Regier, J. C., Kafatos, F. C., Goodflesh, R., and Hood, L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 390-394
2. Thireos, G., and Kafatos, F. C. (1980) Dev. Biol. 78, 36-46
3. MacDonald, R. J., Crerar, M. M., Swain, W. F., Pictet, R. L., Thomas, G., and Rutter, W. J. (1980) Nature 287, 117-122
4. Young, R. A., Hagenbuckle, O., and Schibler, U. (1981) Cell 23, 451-469
5. McKnight, G. S., and Palmiter, R. D. (1979) J. Biol. Chem. 254, 9050-9058
6. LeMeur, M., Glanville, N., Mandel, J. L., Gerlinger, P., Palmiter, R. D., and Chambon, P. (1981) Cell 23, 561-571
7. Selye, H., Cantin, M., and Veilleux, R. (1961) Nature 191, 1076-1078
8. Brown-Grant, K. (1961) Nature 191, 1076-1078
9. Muenzer, J., Bildstein, C., Glesson, M., and Carlson, D. M. (1979) J. Biol. Chem. 254, 5623-5628
10. Muenzer, J., Bildstein, C., Glesson, M., and Carlson, D. M. (1979) J. Biol. Chem. 254, 5629-5634
11. Kumarasamy, R., and Symons, R. H. (1979) Anal. Biochem. 95, 359-363
12. Laemmli, U. K. (1970) Nature 227, 680-685
13. Harding, J. D., Przybyle, A. E., MacDonald, R. J., Pictet, R. L., and Rutter, W. J. (1978) J. Biol. Chem. 253, 7531-7537
14. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408-1412
15. Darnbrough, C., Legon, S., Hunt, T., and Jackson, R. (1973) J. Mol. Biol. 76, 379-403
16. Pelham, H. R. B., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247-256
17. Bonner, W. B., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88
18. Kreil, G. (1981) Annu. Rev. Biochem. 50, 317-48
19. Palmiter, R. D., Gagnon, J., Ericsson, L. H., and Walsh, K. A. (1977) J. Biol. Chem. 253, 6386-6393
20. Furthmayr, H., and Timpl, R. (1971) Anal. Biochem. 41, 510-516
21. Tsang, B. K., Dixon, R. H., and Whittfield, J. F. (1960) J. Cell Physiol. 102, 19-26
22. Butcher, F. R., Rudich, L., E isoler, C., and Nemirovski, M. (1976) Mol. Pharmacol. 12, 862-870
Cell-free translations of proline-rich protein mRNAs.
M A Ziemer, A Mason and D M Carlson

J. Biol. Chem. 1982, 257:11176-11180.

Access the most updated version of this article at http://www.jbc.org/content/257/18/11176

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/18/11176.full.html#ref-list-1