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CYTOSINE-RICH MESSENGER RNA FROM CARROT ROOT DISCS

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Summary: The total poly A^+ RNA from aerated carrot root discs was further fractionated into a cytosine-rich mRNA fraction by oligo (dG) cellulose chromatography. C-rich mRNA was purified at least 10-fold by this procedure and, when translated in wheat germ lysates, codes for 57 and 53 Kdalton peptides. Translation in double label amino acid mixtures indicates that C-rich mRNA codes for proline-rich peptides which may be precursors to the hydroxyproline-rich glycoprotein synthesized in vivo by this tissue.

INTRODUCTION

Hydroxyproline-rich glycoproteins (HRGPs) are found in cell walls throughout the plant kindgom (1, 2), in the medium of suspension cell cultures (3) and as a lectin in members of the Solanaceae family (4-6). Proteolytic peptides derived from HRGPs covalently bound cell wall HRGPs contain polyhydroxyproline (3 to 5 residues long) flanked by serine (1, 7). The potato lectin (5) and Chlamydomonas cell wall (8) HRGPs contain large domains which are predominantly polyhydroxyproline. These HRGPs contain far more polyhydroxyproline than is found in collagen (9). Since hypro arises in plants from proline by posttranslational modification (10), it is coded for by CCX. In chicken Pro-α1(I) collagen mRNA, C occurs in the third position of 69% of pro codons in the sequence

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HRGP: hydroxyproline-rich glycoprotein; hypro: hydroxyproline

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coding for the hydroxyproline-rich, helical region of the protein (11). These observations raise the possibility that plant HRGPs may be translated from a C-rich mRNA.

We have isolated and purified C-rich mRNA from carrot root discs by hybridizing total poly A^+ RNA with oligo (dG) cellulose. This C-rich mRNA codes for peptides of 57 and 53 Kdalton which are proline-rich and are of similar size to the 55 Kdalton precursor to HRGP synthesized in vivo.

MATERIALS AND METHODS

Protein labeling. Tap roots of carrots were aseptically sliced and incubated with 50 µg/ml chloramphenicol or 100 µg/ml penicillin G (12, 13). Tissue was labeled with 10 µCi of L-[2,3,4,5-^{3}H]Pro (100 Ci/mmol) for 1 hr, rinsed free of label and homogenized and extracted as described previously (13). Unhydroxylated precursor was labeled in the presence of 100 µM α,α'dipyridyl, a prolyl hydroxylase inhibitor in plants (10).

RNA extraction and purification. Discs used for RNA extraction (100 gm fresh weight) were aerated for 48 hr and labeled continuously with 250 µCi [^{3}H]-Ado (55 Ci/mmol). The incubation media and label were replaced after 24 hr of incubation. Discs were blotted dry, frozen with $N_{2(\ell)}$, and ground to a fine powder in a stainless steel Waring blender. This powder was thawed in 400 ml 0.2 M glycine (pH 9.0), 30 mM EGTA, 70 mg Proteinase K, 1% SDS, 2 mM DTT, and stirred 1 hr at 37 C. The suspension was chilled on ice, 80 ml of 2 M KCl was added, and centrifuged 10 min at 14,000 rpm in a Sorvall GSA rotor. RNA was precipitated by making the supernatant 70% with ethanol. The poly A^+ RNA was purified as described previously (14). Cytosine-rich RNA was purified by oligo (dG) cellulose (Collaborative Research, Waltham, MA) chromatography using the formamide denaturation steps described by Mozer (14). One-ml fractions were taken of the eluate of the oligo (dG) column until cpm reached background values (5 fractions). Cytosine-rich mRNA was eluted by lowering the NaCl concentration stepwise using 10 mM Tris-Cl (pH 7.6), 1% SDS buffer containing 100 mM NaCl, 100 mM NaCl 60°C, or 10 mM NaCl, 60°C.

In vitro translation. Translation reactions were performed in wheat germ lysates (15) under the conditions described by Mozer (14). Here labeling was done with [2,3,4,5-^{3}H]-Pro (3 µCi per 50 µl reaction mix with total pro equal to 6 µM). In cases where double label in vitro translation was performed, 3 µCi [^{3}H]-Glu (27 Ci/mmol) and 0.3 µCi [U-^{14}C]-Pro (250 mCi/mmol) were used with total glu and pro being 6 µM in the reaction mix. TCA precipitable cpm for reactions were determined as described in Ref. 15.

Electrophoresis. In vivo synthesized proteins and in vitro translation products were analyzed by SDS polyacrylamide gel electrophoresis (16), with slight acrylamide modifications (14) and by pH 4.3-acetate polyacrylamide gel electrophoresis at 7.5% acrylamide (17) which had been made with 8 M urea in the resolving and stacking gels as well as in the sample buffer. Gels were subjected to fluorography at -70 C (18). Rod gels were sliced into 2 mm sections, and digested in scintillation vials with 1 ml Nuclear Chicago solubilizer at 50°C for 2 hr. The slices were cooled, neutralized with 1 ml 0.5 M Tris-Cl and counted in a xylene, triton-X-100 based cocktail.

TABLE 1. Oligo-dG-cellulose chromatography of mRNA from carrot root discs.

NaCl (mM)	Temperature	% of RNA*
500	23	91
100	23	7
100	60	1.5
10	60	0.5

* Determined from cpm of adenosine labeled poly A^+ RNA.

RESULTS

The results of oligo (dG) cellulose chromatography of poly (A) RNA isolated from 48 hr incubated discs is shown in Table 1. Ninety-one percent of the cpm from poly A^+ RNA does not hybridize with oligo (dG) cellulose and elutes at 500 mM NaCl. Lowering the NaCl concentration to 100 mM elutes 7% of the RNA. Small amounts of additional C-rich RNA were eluted at 100 mM NaCl at 60 C and at 10 mM NaCl at 60 C.

The in vitro translation products of RNA fractionated by oligo (dG) cellulose chromatography were analyzed by SDS (Fig. 1) and pH 4.3 (Fig. 2) polyacrylamide gel electrophoresis. The RNA eluting from oligo (dG) cellulose at 500 mM NaCl codes for most of the peptides present in the unfractionated poly (A) RNA (Fig. 1A). Absent, however, are several peptides greater than 50 Kd. RNA eluted at 100 mM and 10 mM NaCl code for two predominant peptides which are 57 and 53 Kd. These translation products are compared with precursor to HRGP synthesized in vivo in carrot discs treated with 100 μM α,α 'dipyridyl (Fig. 1B). The unhydroxylated, unglycosylated precursor to HRGP migrates at 55 Kd in SDS-PAGE (see also Ref. 19). In vitro translation products of carrot mRNA are also compared to HRGP and HRGP precursor in Figure 2. RNA purified by oligo (dG) hybridization codes for fewer peptides than does the total mRNA or RNA passing through the column at 500 mM NaCl. The predominant peptides coded for by C-rich RNA comigrate with

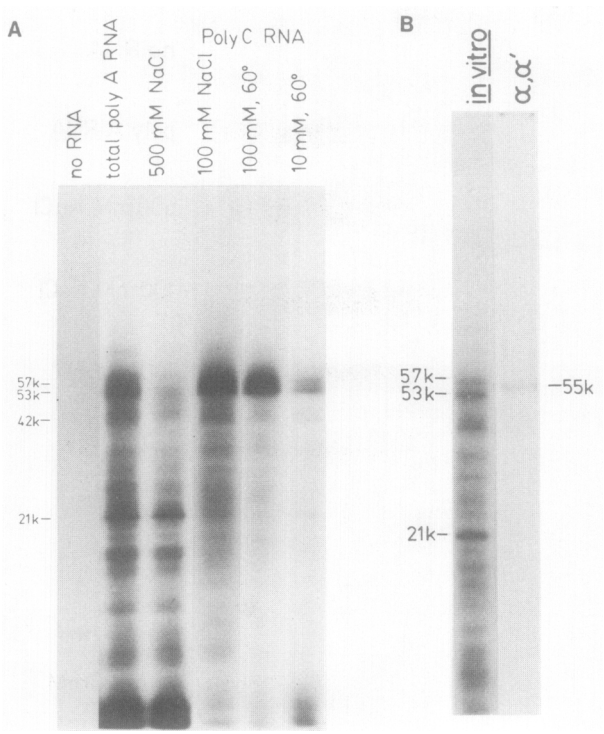

Figure 1. In vitro translation products of poly (A) RNA from carrots analyzed by SDS-polyacrylamide gel electrophoresis. Translation was done in the presence of 3 μCi [^{3}H]-Pro. 1A. Translates of mRNA were fractionated on oligo (dG) cellulose with the fractionation conditions noted in Table 1. 1B. Comparison of the mobility of in vitro translation products of poly (A) RNA (in vitro) and in vivo labeled precursor to HRGP extracted from cell walls of tissue labeled with [^{3}H]-Pro in the presence of 0.1 mM α,α'dipyridyl (α,α').

precursor to HRGP (α,α'). Fully glycosylated HRGP synthesized in vivo does not enter SDS-PAGE gels (see Ref. 13) and migrates slower than its precursor and translation products of C-rich mRNA in pH 4.3 gels.

In Table 2 are shown the results of an in vitro protein synthesis reaction done in the presence of [^{14}C]-pro and [^{3}H]-gln.

TABLE 2. The ^{14}C to ^{3}H ratio of trichloroacetic acid precipitable cpm from in vitro translation products labeled in the presence of 3 μCi ^{3}H-Glu and 0.3 μCi ^{14}C-Pro.

RNA added to reaction	$^{14}C/^3H$
No RNA (endogenous activity)	0.121
Poly A$^+$ RNA	0.242
C-rich mRNA eluted at:	
100 mM NaCl, 23 C	0.471
100 mM NaCl, 60 C	0.588

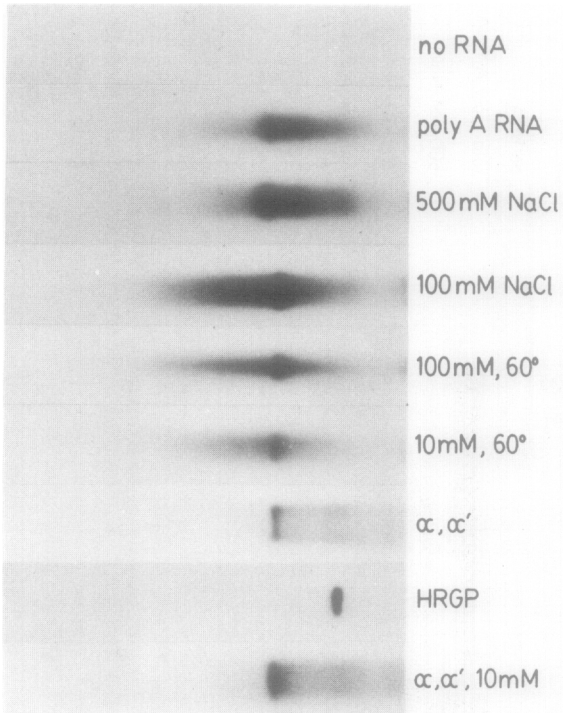

Figure 2. Acidic, pH 4.3 polyacrylamide gel electrophoresis of in vitro translation products of poly (A) and (C)-rich RNA. Translation products labeled with 3 μCi [^{3}H]-Pro. RNA was fractionated as described in Table 1 and the elution conditions are noted above each lane. Also shown is purified HRGP precursor (α,α'), HRGP and the translation products of 10 mM NaCl mRNA and HRGP precursor run in the same lane (α,α', 10 mM).

Translation products of C-rich mRNA are richer in proline, as indicated by the increase in the ^{14}C to ^{3}H ratio, than are the products of the endogenous wheat germ mRNA or the total poly RNA of carrot. When SDS gels of in vitro translation products of mRNA eluted from oligo (dG) cellulose at 100 mM NaCl at 23 C were sliced and counted the ^{14}C to ^{3}H ratio at 57 and 53 Kdaltons averaged 8-fold greater than the average endogenous peptide found in wheat germ (data not shown).

DISCUSSION

Sliced and aerated carrot root discs synthesize an 86 Kdalton hydroxyproline-rich glycoprotein after 10 h of incubation (12). This glycoprotein contains 50% hypro in its 55 Kd peptide backbone; far more than any other protein known (13). If hydroxyproline is

randomly distributed in this peptide, some polyhydroxyproline would be expected to occur. In the best studied cases, hydroxyproline occurs predominantly (8) or exclusively (2, 5, 7) as polyhydroxyproline in plant derived HRGPs. It was for these reasons we chose aerated carrot root discs as a model system for isolating a C-rich mRNA.

The results presented here suggest that the precursor to carrot HRGP is coded for by C-rich mRNA. Two proline-rich peptides were translated in vitro from C-rich mRNA isolated from aerated carrot discs (Table 1, 2; Figure 1A). These peptides have molecular weights by SDS-PAGE of 57 and 53 Kdaltons, which are similar to the 55 Kdalton precursor peptide produced in vivo in α,α'dipyridyl-treated carrot tissue (Fig. 1B, Ref. 19). The electrophoretic mobility of the translation products of C-rich mRNA are also similar to the precursor of HRGP in the pH 4.3 gel system, an electrophoresis system which was previously used to purify and characterize the glycoprotein (Fig. 2, Ref. 12). Finally, the in vitro translation products of C-rich mRNA have an extremely high proline content compared to endogenous wheat germ mRNA or the proline to glutamate ratio expected from an "average" protein (20).

Preliminary experiments also indicate that C-rich mRNA is absent from fresh carrot root discs which do not synthesize HRGP. More definitive identification of the in vitro translation products of C-rich carrot mRNA has been sought unsuccessfully by attempting to immunoprecipitate products with antibody raised to HRGP. This failure may be due to the structural dissimilarity of the precursor and HRGP since the former undergoes extensive hydroxylation, glycosylation and helix formation to become the latter. Critical identification of the in vitro translation products of C-rich mRNA must await the production of antibody to deglycosylated or unhydroxylated HRGP antigens or peptide mapping studies.

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