POLYADENYLATION OF 35S RNA IN VITRO BY A CYTOPLASMIC PARTICULATE FRACTION FROM MOUSE MYELOMA

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SUMMARY

An ATP incorporating activity was found to be associated with cytoplasmic particulate fractions prepared from MOPC-104E tumors (a mouse plasmacytoma). The product was shown to be poly(A) by its chromatography pattern on oligo(dT) cellulose columns, and the endogenous primers included a 35S RNA species as analysed by Cs_2SO_4 density centrifugation and polyacrylamide gel electrophoresis. The labeled poly(A) was estimated to have a chain length of 200 nucleotides by gel electrophoresis, and hydrolysis of the poly(A) yielded approximately 100 AMP residues for every adenosine residue.

INTRODUCTION

It is generally accepted that gene expression requires the nuclear synthesis of an RNA transcript, the 3'-terminal polyadenylation and specific phosphodiester bond cleavage of that transcript (RNA processing), the transport of the processed transcript to the cytoplasm and the utilization of it as a template directing the synthesis of one or more functional polypeptides. Although the nucleus is usually implicated as the site of polyadenylation, cytoplasmic polyadenylation is known to occur in some systems, e.g. in fertilized sea urchin eggs [1] and in some animal viruses that are thought to replicate exclusively in the cytoplasm [2-4]. Nevertheless, cytoplasmic polyadenylation seems to be the exception rather than the rule. Diez and Brawerman [5] recently showed that in Chinese hamster and mouse sarcoma 180 ascites cells, elongation of poly(A) chains in the cytoplasm is primarily restricted to turnover of the terminal 7-8 nucleotides.

In this study, we present data showing that cytoplasmic particulate fractions from a mouse plasma tumor (MOPC-104E) were able to synthesize poly(A) *in vitro*. The endogenous primers contained 35S RNA and the poly(A) synthesized *in vitro* had an average chain length of 100. This poly(A)-synthesizing activity copurified with intracisternal type-A particles on isopycnic-sucrose gradients and might be indigenous to these particles. This is strengthened by the recent finding of a 35S, poly(A)-containing RNA associated with type-A particles [6].

MATERIALS AND METHODS

Preparation of cytoplasmic particulate fractions - MOPC-104E tumors maintained subcutaneously in BALB/C mice were homogenized in isotonic buffer containing 0.25 M sucrose, 50 mM Tris HCl, pH 7.6, 25 mM KCl and 5 mM $MgCl_2$ at 4°C. After removal of nuclei by centrifugation, the procedure of Kuff *et al.* was followed to obtain a membrane bound particulate fraction by detergent treatment of the 12,000 xg pellet. The 12,000 xg supernatant was centrifuged at 100,000 xg to yield a free cytoplasmic particulate fraction. Both fractions were centrifuged isopycnically on 30-60% sucrose gradients.

Polymerase assay - The assay mixture for poly(A) polymerase contained per ml: 50 µmoles Tris-HCl pH 7.4, 2 µmoles NaF; 50 µg phosphocreatine kinase; 4 µmoles phosphocreatine; 5 µmoles dithiothreitol, 1.5 µmoles $MnCl_2$, 1 mC [^{3}H]ATP (15 C/mmole) and 0.15% Triton X-100 (final concentration). The RNA-dependent DNA polymerase assay mixture contained, per ml: 50 µmoles Tris-HCl, pH 8.3, 200 µmoles $Mg(OAc)_2$; 20 µmoles dithiothreitol, 1 mC [^{3}H]TTP (47 C/mmole); 100 µg oligo(dT)·poly(A) (P.L. Biochemicals) and 0.3% Triton X-100. All reactions were carried out at 37° for 60 min. and terminated by addition of 10% trichloroacetic acid.

Oligo(dT)-cellulose chromatography - Oligo(dT)-cellulose (T3, Collaborative Research) chromatography was carried out at room temperature as described [21]. Samples were applied in buffer containing 0.5 M NaCl-0.01 M Tris-hydrochloride, pH 7.5, to an oligo(dT)-cellulose column previously equilibrated with the same buffer. After elution of the non adsorbed material by washing with five times the bed volume of the application buffer, the material retained was either eluted directly with H_2O, or where stated, was just eluted with a buffer of intermediate ionic strength (0.1 M NaCl, 10 mM Tris HCl, pH 7.5).

Determination of AMP/adenosine ratio of the labeled reaction product - After digestion of the reaction product with ribonuclease, the poly(A) was reisolated by fractionation on oligo(dT)-cellulose. The H_2O eluate was hydrolyzed in 0.1 M NaOH at 50° for 16 hours in a sealed ampoule. The hydrolysate was then spotted on Whatman #1 paper, neutralized with acetic acid fumes and electrophoresed at 3000 KV for 1 hour in pyridine-acetate buffer, pH 3.5. Strips of 1 cm were cut and assayed for radioactivity by scintillation counting. The strips containing [^{3}H] adenosine radioactivity were counted for 40 min.

RESULTS and DISCUSSION

Tissues from the tumors were disrupted and processed according to the procedure described by Kuff *et al.* [7] to obtain a 100,000 xg pellet from a triton-

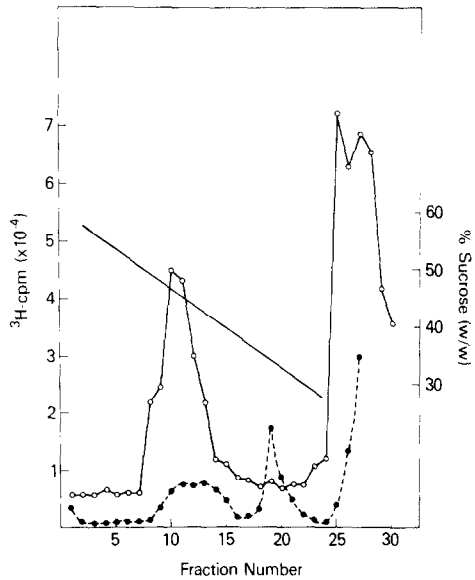

Fig. 1 -- Nucleic acid polymerase activities of cytoplasmic particulate fractions on linear sucrose gradients.

The 100,000 xg pellet from post-mitochondrial supernatant was fractionated isopycynically by centrifugation in 30-60% sucrose gradients [7]. Conditions for poly(A) polymerase and RNA-dependent DNA polymerase assays are given in Materials and Methods. The 100,000 xg pellet from a triton-treated mitochondrial fraction [7] yielded a similar profile (not shown). No DNA-dependent RNA polymerase activity was found, judged by absence of incorporation of [^{3}H]UTP under appropriate ionic conditions (not shown). O = incorporation of [^{3}H]ATP, ● = incorporation of [^{3}H]TTP.

treated, crude mitochondrial fraction. In addition, particulate material was obtained from the post-mitochondrial supernatant by centrifugation (100,000 xg) without triton treatment. When these fractions were centrifuged isopycnically on linear, 30-60% sucrose gradients and assayed for incorporation of [^{3}H]ATP across the gradients, both fractions yielded a region of activity at an average density of 1.22 gm/ml (47% w/w sucrose, Fig. 1). The ATP-incorporating activity at high density was coincident with a DNA polymerase activity stimulated by oligo(dT)·poly(A), an activity that has been ascribed to intracisternal A-particles [8,9]. The DNA polymerase activity at 1.15 gm/ml (34% sucrose w/w) might be associated with mitochondria or other cell organells.

The ATP incorporation reaction was further characterized and Table 1 summarizes the results. The presence of excess unlabeled CTP, GTP and UTP neither stimulated nor inhibited the reaction, thus making unlikely RNA polymerase or ter-

Table 1. Poly(A) Polymerase Activity of Cytoplasmic Particulate Fractions Banding at 1.22 g/ml in Sucrose

Reaction Conditions	Incorporation of [^{3}H]ATP (% of standard reaction)
Standard reaction	100%
+ GTP, UTP, and CTP (120 μM each)	100%
+ 3'dATP (10 μM)	30%
- triton	50%
post-incubation with RNase A (20 μg/ml, 37°/1 hr.)	80%
post-incubation with alkali (.3 M KOH, 100°/20')	0%

Standard reaction mixture contained 66 μM [^{3}H]ATP and other constituents as described in Legend to Figure 1. Ribonuclease incubation was carried out in 0.3 M NaCl, 0.03 M sodium citrate pH 7.0. Incorporation of the standard reaction was 6000 cpm.

minal transferase-like activity [10]. Cordycepin triphosphate (3'-dATP) at one-sixth the concentration of [^{3}H] ATP inhibited 70% of the incorporation. Omission of triton reduced the radioactivity incorporated by 50%. In addition, the reaction product was resistant to RNase treatment under conditions that do not degrade synthetic poly(A), but was sensitive to alkali. These results are consistent with incorporation of [^{3}H]ATP into poly(A) by a cytoplasmic particle of density 1.22 gm/ml in sucrose.

The reaction product was purified by SDS-phenol-chloroform as described elsewhere [11] and further analysis revealed the following properties:

i. The Product is RNA

As shown in Fig. 2, more than 90% of the radioactivity showed a buoyant density of 1.61 gm/ml in Cs_2SO_4, indicating that [^{3}H]ATP was incorporated into RNA.

ii. The Reaction Product Binds Oligo(dT)-Cellulose

Depending on the particular preparation, 20-60% of the total ATP incorporated was retained on oligo(dT)-cellulose columns in 0.5 M NaCl. Subsequent

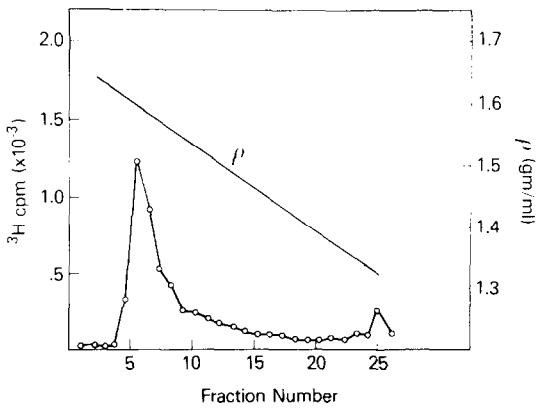

Fig. 2 -- Buoyant density of the reaction product on polyacrylamide gels.

After extraction with SDS-phenol-chloroform, the reaction product was mixed with an equal volume of saturated Cs_2SO_4 solution containing 10 mM Tris-HCl, pH 7.2, 2 mM EDTA, 0.1% sarkosyl (final density = 1.50 gm/ml) and centrifuged at 35,000 rpm for 60 hours at 15° in a SW 50·1 rotor.

elutions with 0.1 M NaCl and H_2O were performed and the results are shown in Table 2. Ratios of the H_2O eluate increased with time of incubation, suggesting elongation of the poly(A) chain in vitro. When the H_2O eluate was rechromatographed on oligo(dT)-cellulose, 90% of the radioactivity again bound to the column. The same result was obtained after heat treatment or exposure to RNase in high salt before chromatography. The results of i. and ii. are consistent with covalent linkage of the incorporated [^{3}H]ATP to a poly(A)-containing RNA.

iii. The RNA Primers Contain a 35S RNA

After partitioning the product of a 20 min. reaction in vitro on an oligo(dT)-cellulose column, the poly(A)-containing radioactivity eluted with H_2O was examined by electrophoresis in polyacrylamide gels. Fig. 3 illustrates the electrophoretic mobilities of the native, denatured, and ribonuclease-digested, reaction product. In one experiment, a major portion of the incorporated radioactivity migrated as a 35S peak on 2% polyacrylamide gels before and after heat treatment, (Fig. 3a and 3b), indicating that ATP was incorporated into a 35S poly (A)-containing RNA by covalent linkage. The relative amount of radioactivity in 35S RNA varied from preparation to preparation. We attribute this variation to

Table 2. Oligo(dT)-Cellulose Chromatography of the Poly(A) Polymerase Reaction Product

	cpm in flow-through and wash	cpm eluted		% bound cpm eluted in H_2O
	0.5 M NaCl	0.1 M NaCl	H_2O	
(a) 5' reaction	1255	468	219	32
(b) 20' reaction	2132	411	753	65
(C) 45' reaction	3058	553	1624	75
(d) Rechromatography of H_2O eluate from (c)	257	ND	1889	89
(e) Same as (d), after heating to 100°, 5'	230	ND	1457	87
(f) same as (e), after digestion with 20 µg/ml RNase A in .3 M NaCl, 30° for 3'	250	ND	900	79

Conditions for chromatography were essentially as those described by Swan et al. [21]. (a), (b) and (c) were loaded in 0.5 M NaCl and washed with 5 bed volumes each of 0.5 M NaCl, 0.1 M NaCl and H_2O in succession. For samples (d) (e) and (f) the intermediate elutions with 0.1 M NaCl were omitted. ND = not determined.

degradation of the RNA during incubation and purification. To determine whether the ATP is indeed incorporated into poly(A) itself, we analyzed a RNase A and T_1 limit digest of the product on 5% polyacrylamide gels. As shown in Fig. 3c, two major peaks could be identified, with R_f values of 0.5 and 1 relative to a 4S marker. These corresponded to poly(A) sizes of 200 and 80 nucleotides respectively. The size of the poly(A) increased with time during *in vitro* reaction, reaching a maximum at 10 min. and remaining unchanged to 60 min. of reaction (not shown). These sizes must be considered the sum of the length of the labeled poly(A) synthesized *in vitro plus any adjacent unlabeled* poly(A) synthesized *in vivo*.

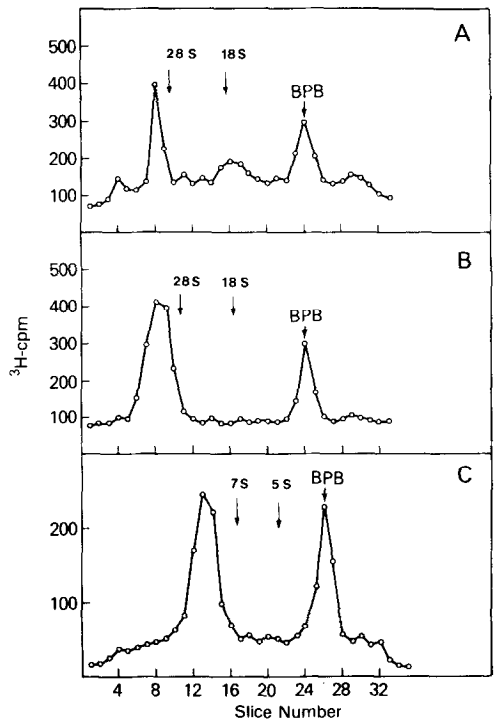

Fig. 3 -- Electrophorectic mobility of the reaction product on polyacrylamide gels.

Conditions for preparation of 2% and 5% polyacrylamide gels and electrophoresis in buffer containing 0.5% SDS were those described by Bishop et al [2]. A) native product electrophoresed in 2% polyacrylamide B) native product heated to 100° for 10 min. electrophoresed in 2% polyacrylamide C) native product heated to 100° for 10 min, then treated with 20 µg/ml RNase A. In C, 4S RNA comigrated with bromophenol blue (BPB) marker. 25 units/ml RNase T_1 in 0.3 M NaCl at 37° for 30 min, then electrophoresed in 5% polyacrylamide. Bromophenol blue (BPB) was used as a dye marker.

The average number of adenosine residues added to each poly(A) chain in vitro was determined by measuring the AMP adenosine ratio of the labeled product after alkaline hydrolysis (Fig. 4), assuming that only adenosine monophosphate residues were added and that they were appended to the 3'terminus. The average AMP/adenosine ratio determined by electrophoresis of the hydrolyzed reaction product was 90, 150, and 60 in three separate experiments. Treatment of the poly(A) with alkaline phosphatase before hydrolysis did not alter the ratio (not shown). Thus, it is unlikely that the high ratio results from nucleolytic activity on the polyadenylated residues. Since the extent of polyadenylation of different RNA molecules may

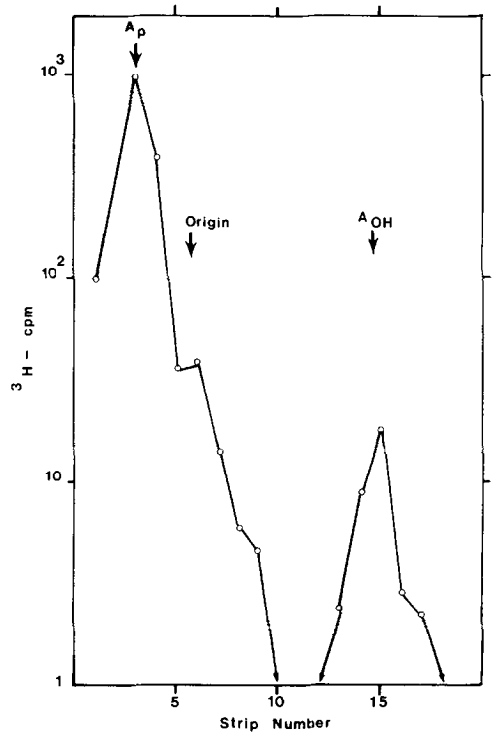

Fig. 4 -- AMP/adenosine ratio of the reaction product following alkaline hydrolysis.

Digestion of the product and subsequent paper electrophoresis are described in Materials and Methods. The radioactivity in regions corresponding to AMP or adenosine markers were summed (in set). The positive pole is at the left of the figure. 1800 cpm were recovered in the adenosine region. The calculated length of the poly(A) added in vitro was ninety-one nucleotides (1800/20 + 1).

be heterogeneous at the start of the in vitro reaction, this AMP/adenosine ratio should give a lower limit of (AMP) residues that can be added in vitro. The phenomenon is therefore different from the "turnover" of the 7-8 3'proximal residues described by Diez and Brawerman [5]. Although the reaction in vitro may not reflect the in vivo event, the fact that polymerization stops after the poly(A) reaches 200 residues (Fig. 3c) indicates a similar regulatory mechanism both in vivo [6] and in vitro.

Since the poly(A) polymerase activity copurified with intracisternal A-particles on isopycnic sucrose gradients and since over 20% of the RNA primer molecules are 35S, the activity may be a constituent of the type-A particles. It has

been proposed that cytoplasmic polyadenylation may be common to processing of RNA of embryos and of type-B and type-C RNA viruses [12,13]. That RNA of early sea urchin embryos can be cytoplasmically polyadenylated has been documented [1,14-17]. Type-A particles resemble type-B and type-C RNA viruses in that both contain 1) reverse transcriptase [7,8] and 2) high molecular weight RNA [6,9,18] terminating in a poly (A) tract 200 adenosine residues long [6,19]. Furthermore, intracisternal type-A particles appear to be a cytoplasmic component of normal, early mouse embryos [20]. The concept that cytoplasmic polyadenylation is a common feature of the expression of embryonic and type-A, -B and -C virus genes is therefore strengthened.

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