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Isolation and characterization of cytoplasmic poly(A) polymerase from cryptobiotic gastrulae of *Artemia salina*

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Poly(A) polymerase has been purified to near homogeneity from the cytoplasm of *Artemia salina* cryptobiotic gastrulae by ion-exchange chromatography on DEAE-cellulose, DEAE-Sepharose CL-6B and phosphocellulose P11, gel filtration on CL-Sepharose 6B, affinity chromatography on poly(A)-Sepharose 4B and ATP-agarose. The enzyme is fully dependent on exogeneous oligo(riboadenylic acid) and is free of any nuclease or other enzyme activities. In standard assay conditions the enzyme preparation has a specific activity of 5.6 μmol AMP · h^{-1} · (mg protein)$^{-1}$.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis reveals the presence of only two proteins with M_r 94000 and 70000. The M_r-70000 protein has been identified as poly(A) polymerase.

The enzyme is exclusively activated by Mn^{2+}. Addition of Ca^{2+}, Mg^{2+}, Zn^{2+}, NH_4^+, K^+ or Na^+ inhibits the enzymatic reaction. The activity is specific for ATP and competitive inhibition is observed in the presence of other ribonucleoside 5′-triphosphates. AMP incorporation is time-dependent and is increased non-linearly with protein and primer concentration.

In higher eukaryotes most of the hnRNP is polyadenylated post-transcriptionally at the 3′ terminus to a length of 200–250 nucleotides. Polyadenylation is initiated after cleavage of the primary transcripts [1, 2] as transcription proceeds beyound the poly(A) addition site [3, 4] believed to be situated about 20 nucleotides downstream from the A-U/A-U-A-A-A sequence [5, 6]. The function of the poly(A) · protein complex is not clear and several hypothesis have been proposed: (a) regulation of hnRNA splicing [7, 8] and selection of the 3′-terminal mRNA regions during mRNA biogenesis resulting in a regulation of gene expression [1–3]; (b) transport to the cytoplasm may be mediated by poly(A) binding proteins [9]; (c) stabilization of mRNA if longer than 21 nucleotides [10–13]; (d) regulation of translation [7].

The presumed functional significance of the 3′-terminal poly(A) sequence has stimulated the study of enzymes involved in poly(A) metabolism. Recently several factors have been found to regulate this phenomenon. Enzymes capable of adding poly(A) sequences *in vitro* to single-stranded RNA have been found in nuclei, mitochondria, microsomes, ribosomes and the cytosol [14, 15]. Nuclear poly(A) polymerase is thought to adenylate *de novo* spliced primary transcripts [16, 17]. In embryogenesis the nuclear poly(A) polymerase activity is increased with a concomitant decrease in activity of the cytoplasmic enzyme [18–20]. The function of cytoplasmic poly(A) polymerase is still unclear. It is known that poly(A) is hydrolyzed to a heterogeneous but more stable steady-state length after leaving the nucleus. This stable poly(A) length may protect mRNA against nuclease attack [7]. Cytoplasmic polyadenylation also seems to occur after reactivation of cell metabolism [19, 21]. The latter elongation is followed by a partial hydrolysis of the poly(A) sequence and transfer of mRNA into polysomes [10, 22, 23]. Both processes indicate a close relationship between adenylating and deadenylating enzyme activities. A poly(A) polymerase/poly(A) endoribonuclease IV complex is found in the nucleus [24] and in the cytoplasm [25] of calf thymus. A 2′,3′-poly(A) exoribonuclease is also discovered [26].

In an attempt to elucidate the function of polyadenylation of mRNA by poly(A) polymerase a purification procedure of the enzyme from the cytoplasm of *Artemia salina* cryptobiotic gastrulae has been developed. A preliminary report of this work was presented at a meeting of the Belgian Biochemical Society [27].

EXPERIMENTAL PROCEDURES

Materials

DEAE-cellulose DE52, phosphocellulose P11, GF/C glass-fiber filters, DE81 filter paper from Whatman Biochem Ltd (Maidstone, Kent, UK); poly(A)-Sepharose 4B, CNBr-activated Sepharose 4B, CL-Sepharose 6B, Sephacryl S-200, DEAE-Sepharose CL-6B, low-M_r marker proteins, blue dextran 2000 from Pharmacia Fine Chemicals (Uppsala, Sweden); calf liver tRNA from Boehringer (Mannheim, FRG); ATP, CTP, GTP, UTP, calf thymus DNA, phospho*enol*pyruvate, poly(A), ATP-agarose, catalase, bovine serum albumin, alcohol dehydrogenase, cytochrome *c*, ferritin, chymotrypsin from Sigma Chem. Comp. (St Louis, MO, USA); [^{3}H]ATP, [^{3}H]UTP, [^{3}H]NAD, [^{3}H]poly(A) from Amersham Int (Buckinghamshire, UK); $(A)_4$ from P. L. Biochemicals GmbH (St Goar, FRG); *Artemia salina* cryptobiotic embryos were obtained from the *Artemia* Reference Center, University of Ghent (Belgium); hydroxyapatite from Bio-Rad Labs (Richmond, CA, USA).

Abbreviations. $(A)_4$, adenylyl(3′-5′)adenylyl(3′-5′)adenylyl(3′-5′)adenosine; ADP-ribose, adenosine(5′)diphospho(5)-β-D-ribose; hnRNP, heterogeneous nuclear ribonucleoprotein; mRNP, messenger ribonucleoprotein.

Enzyme. Poly(A) polymerase (EC 2.7.7.19).

Buffer

pH of buffers was measured at 20°C. Buffer A: 50 mM Tris pH 8.6, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 5% (v/v) glycerol; buffer B: 20 mM potassium phosphate pH 5.8, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol; buffer C: 100 mM potassium phosphate pH 5.8, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol; buffer D: 10 mM Hepes pH 7.5, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol; buffer E: 50 mM Tris pH 8.6, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol; buffer F: 50 mM Tris pH 8.6, 100 mM NH_4Cl, 20 mM 2-mercaptoethanol, 50% (v/v) glycerol; buffer G: 25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 300 mM sucrose, 15% (w/v) Ficoll 400, buffer H: 20 mM Tris/HCl pH 7.0, 250 mM KCl, 6 mM magnesium acetate.

Fractionation

Nuclei from 50 g (dry weight) of cryptobiotic gastrulae of *Artemia salina* were prepared in buffer G. Gastrulae were incubated in sea water for 30 min at 4°C and subsequently washed with distilled water and with buffer G. The washed gastrulae were ground in a mortar at 4°C. The homogenate was diluted with 150 ml of buffer G in the presence of 1% Nonidet P40. After filtration through cotton-wool, nuclei, mitochondria and postmitochondrial supernatant were obtained by centrifugation at $2000 \times g_{max}$ for 10 min and at $20500 \times g_{max}$ for 20 min respectively. Criteria used to check the efficiency of the fractionation were: (a) counting of nuclei with a microscope, (b) amount of DNA measured by staining with ethidium bromide [28] and isolated as described by Cruces et al. [29] and (c) activity of DNA-dependent RNA polymerase and poly(ADP)-ribose polymerase assayed as described below. The amount of nuclear poly(A) polymerase activity was measured after lysis of nuclei and partial purification of the enzyme. Complete lysis of nuclei was obtained after extensive dialysis against buffer A. Lysed nuclei were centrifuged at $8000 \times g_{max}$ for 15 min and the pellet washed with buffer A containing 800 mM NH_4Cl and 1% (v/v) Nonidet P40. Fractions enriched in poly(A) polymerase activity were obtained by ion-exchange chromatography on DEAE-cellulose. Nuclear, mitochondrial and postmitochondrial fractions were diluted to 50 mM NH_4Cl and were applied to DEAE-cellulose previously equilibrated with buffer A and 1% (v/v) Nonidet P40. Bound material was eluted with buffer A containing 120 mM NH_4Cl. After dialysis against buffer A, fractions were assayed for poly(A) polymerase activity. Cytoplasmic poly(A) polymerase was purified from the postmitochondrial supernatant of 600 g (dry weight) of cryptobiotic gastrulae. The homogenate prepared as described previously [30] was diluted with 2 l of buffer A, stirred for 10 min and centrifuged at $4200 \times g_{max}$ for 30 min. The postmitochondrial supernatant was obtained after filtration through cheese cloth and centrifugation at $32000 \times g_{max}$ for 30 min.

Chromatographic procedures

Ion-exchange chromatography on DEAE-cellulose, phosphocellulose P11 and DEAE-Sepharose CL-6B was as described by the manufacturer. CL-Sepharose 6B and Sephacryl S-200 columns were calibrated in buffer B containing 200 mM KCl and buffer A containing 500 mM NH_4Cl respectively with ferritin (M_r 450000), catalase (M_r 250000), alcohol dehydrogenase (M_r 73000), bovine serum albumin (M_r 68000), chymotrypsin (M_r 25000) and cytochrome *c* (M_r 12500). The void volume was measured with blue dextran 2000.

High-resolution liquid chromatography was done on a Ultro Pac TSK-63000 SW column. Flow rate of the column was 640 µl/min. The column was calibrated with catalase (M_r 250000), lactate dehydrogenase (M_r 140000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000) and carbonic dehydrogenase (M_r 30000).

Assays

Poly(A) polymerase assays were in 200 mM Tris pH 8.4, 0.4 mM [^{3}H] ATP (spec. act. 15 cpm/pmol ATP), 20 mM NH_4Cl, 0.9 mM $MnCl_2$ and 7 µM $(A)_4$. Nuclease assays were in 200 mM Tris pH 8.4, 40 µg [^{3}H] poly(A) (spec. act. 0.15 cpm/pmol AMP), 1 mM $MnCl_2$ or $MgCl_2$ and 20 mM NH_4Cl. DNA-dependent RNA polymerase assays were in 50 mM Tris pH 7.8, 0.36 mM ATP, CTP and ATP, [^{3}H] UTP (spec. act. 18 cpm/pmol), 10 mM phospho*enol*pyruvate, 90 mM KCl, 3 mM $MgCl_2$, 40 mM ammonium sulphate and 2.7 µg calf thymus DNA unless crude nuclear fractions were assayed. tRNA:nucleotidyl transferase assays were in 200 mM Tris pH 9.0, 0.36 mM [^{3}H] ATP (spec. act. 15 cpm/pmol ATP), 20 mM NH_4Cl, 4 mM $MnCl_2$ and 17 µM tRNA. Hydrolysis assays of polynucleotide phosphorylase were in 200 mM Tris pH 7.8, 40 µg [^{3}H] poly(A) (spec. act. 0.15 cpm/pmol AMP), 10 mM $MgCl_2$, 300 mM KCl, 20 mM potassium phosphate and 1 mM EDTA. Poly(ADP)-ribose polymerase activities were assayed as described by Oikawa et al. [31]. Protein kinase activities were assayed as described by Thoen and Slegers [32]. Protease assays were in 200 mM Tris pH 8.6, 10 µg hide powder azure and 40 mM NH_4Cl.

The assay mixtures (100 µl) for all except protease were incubated at 30°C for 60 min. The 100-µl assay for protease activity was incubated for 24 h. The reactions were stopped by addition of 1 vol. 10% (w/v) trichloroacetic acid and 2% (w/v) sodium pyrophosphate. After 15 min at 0°C the precipitate was filtered on GF/C glass-fiber filters. After washing with 5 ml 5% (w/v) trichloroacetic acid, 5 ml distilled water and 5 ml ethanol, the filters were dried. In the endonuclease assay 60 µl was spotted on DE81 paper previously washed with 4 M urea, 2 M NaCl, 1 mM EDTA. The DE81 paper was washed three times for 10 min with 5 ml of the same solution, 5 ml distilled water and 5 ml ethanol respectively. After drying, the residual radioactivity was counted in a Packard Tri-Carb 2450 scintillation spectrometer. The protease assay mixture was centrifuged at $8000 \times g_{max}$ for 15 min and the absorbance measured at 595 nm.

Protein determination

The protein content was measured by the method of Lowry et al. [33] using bovine serum albumin as a standard or by absorbance measurements at 280 nm.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was as described by Weber and Osborn [34]. Proteins were precipitated with 10% (w/v) trichloroacetic acid, washed with ethanol and ether, dried and dissolved in 20 µl sample buffer (10 mM Tris, 2% w/v sodium dodecyl sulphate, 20% v/v glycerol and 30 mM 2-mercaptoethanol). Gels were stained

overnight in 50% (v/v) methanol, 7.5% (v/v) acetic acid and 0.1% (w/v) Coomassie brilliant blue R250.

RESULTS

Cellular localization of poly(A) polymerases

Nuclei, mitochondria and postmitochondrial supernatant were prepared in buffer G as described in Experimental Procedures. The integrity of approximately 70–80% of the nuclei was demonstrated by microscopic observations, by assays of DNA-dependent RNA polymerase and poly(ADP)-ribose polymerase, and by measurement of the DNA content (Table 1). Measurements of the poly(A) polymerase activity in the nuclear, mitochondrial and postmitochondrial fractions indicated that the activity was located in the postmitochondrial supernatant. No activity was observed in the mitochondrial and nuclear fractions if $(A)_4$ was used as primer. The enzyme activity was not associated with cytoplasmic ribonucleoproteins as demonstrated by assays on ribosomes and non-polysomal poly(A)-containing mRNP.

Table 1. *Integrity of nuclei and subcellular localization of poly(A) polymerase*
Nuclei were estimated by microscopic observation. Poly(A) polymerase activity was measured with $(A)_4$ as primer. n.d. = not detectable

Component	Amount in		
	nuclear fraction	mitochondrial fraction	postmitochondrial supernatant
	% total		
Nuclei	80	20	n.d.
DNA	73	13	14
DNA-dependent RNA polymerase	84	16	n.d.
Poly(ADP)-ribose polymerase	68	7	25
Poly(A) polymerase	1	n.d.	99

Purification of poly(A) polymerase

Step 1. Ion-exchange chromatography on DEAE-cellulose. The postmitochondrial superantant of cryptobiotic gastrulae of *Artemia salina* was prepared in buffer A. Complete binding to DEAE-cellulose was only obtained after extensive dialysis against buffer A. Bound material was eluted with a linear NH_4Cl gradient in buffer A (Fig. 1). Poly(A) polymerase activity was eluted in a sharp peak at 80 mM NH_4Cl. In contrast, the elution of the poly(A)-degrading enzymes was very broad. The enzyme-containing fractions were pooled. This pool contained 25% of the initial amount of protein and the exonuclease and endonuclease activity was reduced to 20% and 13.5% respectively (Table 2).

Step 2. Ion-exchange chromatography on DEAE-Sepharose CL-6B. Pooled fractions of step 1 were diluted with buffer A to a final salt concentration of 50 mM and loaded on DEAE-Sepharose CL-6B. All the AMP incorporating activity was bound to the column. The elution of proteins was performed with a pH gradient from buffer A (pH 8.6) to buffer B (pH 5.8) in the presence of 50 mM NH_4Cl and KCl respectively (Fig. 2). Although the enzyme was unable to bind to DEAE-Sepharose CL-6B at a pH below 8.0 [15], it was not readily eluted from the column. In order to avoid unnecessary dilution, the enzymatic activity was eluted from the column in buffer C. Fractions were assayed for nuclease activity. Although poly(A) polymerase and nuclease activity coincided, the exonuclease and endonuclease activities were further decreased to 5% and 4%, respectively, of the value measured in the postmitochondrial supernatant (Table 2). After this purification step, the pooled fractions (Fig. 2) contained less than 2% of the initial protein and the specific activity of poly(A) polymerase was increased 80-fold.

Step 3. Gel-filtration on CL-Sepharose 6B. Pooled fractions indicated in Fig. 2 were concentrated to 5% of the column volume by dialysis for 2 h against 1 l buffer B containing 200 mM KCl and 95% (v/v) glycerol and chromatographed on a CL-Sepharose 6B column (Fig. 3). Fractions were assayed for AMP incorporation and poly(A) degradation. The poly(A) polymerase as well as the exonuclease activity chromatographed in a sharp peak. The endonuclease activity was more heterogeneously distributed. This step removed more than 95% of the endonuclease activity. Less than 0.1% of the initial endonuclease activity was still present in the pooled fractions. The exonuclease activity of step 2 was

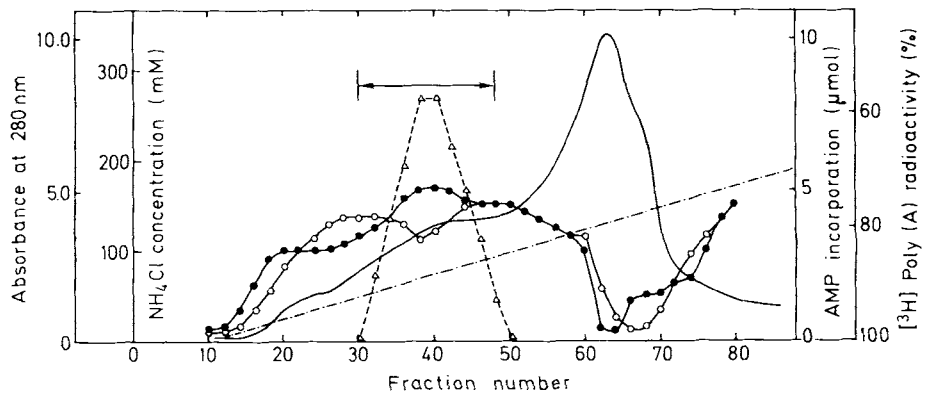

Fig. 1. *DEAE-cellulose ion-exchange chromatography.* A DEAE-cellulose column (5 × 15.5 cm) was equilibrated with buffer A. Postmitochondrial supernatant dialyzed against the same buffer was applied to the column and the bound material was eluted with a salt gradient of 0–200 mM NH_4Cl (–·–·–) in buffer A (2 × 500 ml). Fractions of 13 ml were collected and assayed for AMP incorporation (△----△) and poly(A) degradation by endonuclease (●——●) and exonuclease (○——○) as described in Experimental Procedures. Absorbance at 280 nm (——). Pooled fractions are indicated

Table 2. *Purification of poly(A) polymerase from* Artemia *cryptobiotic gastrulae*
These results are mean values obtained from at least 15 purification experiments. An error of ± 5% has to be taken into consideration. n.d. = not detectable

Fraction	Protein	Total poly(A) polymerase activity	Specific activity	A_{260}/A_{280}	Total nuclease activity	
					exonuclease	endonuclease
	mg	μmol AMP · h^{-1} (%)	nmol AMP · h^{-1} · mg protein^{-1}		μmol AMP · h^{-1} (%)	
Postmitochondrial supernatant	38000.0	140 (100)	4	1.40	9400 (100.0)	9700 (100.0)
DEAE-cellulose	3700.0	235 (168)	65	0.79	1900 (20.0)	1300 (13.5)
DEAE-Sepharose CL-6B	730.0	223 (159)	306	0.98	475 (5.0)	390 (4.0)
CL-Sepharose 6B	350.0	150 (107)	429	1.22	460 (5.0)	8 (0.1)
Phosphocellulose P11 (a)	240.0	137 (98)	571	1.38	170 (2.0)	n.d.
(b)	17.4	33 (24)	1597	2.21	144 (1.5)	n.d.
Poly(A)-Sepharose 4B unbound fraction	9.0	31 (22)	3445	2.07	92 (1.0)	n.d.
ATP-agarose	7.2	25 (18)	3470	1.94	n.d.	n.d.
DEAE-cellulose	3.6	20 (14)	5560	1.83	n.d.	n.d.

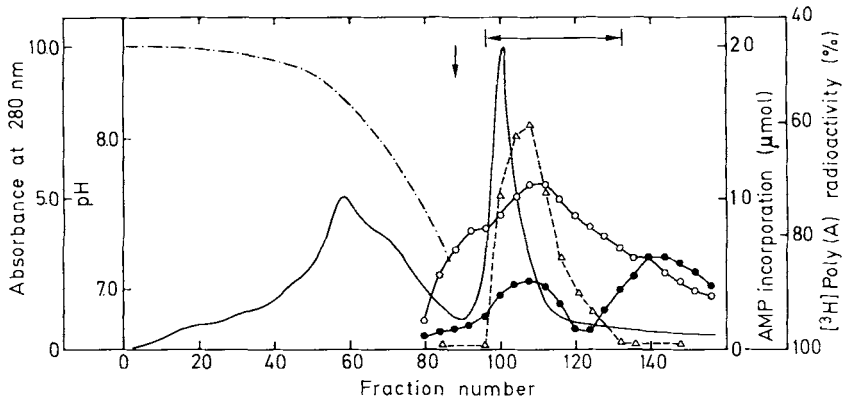

Fig. 2. *DEAE-Sepharose CL-6B ion-exchange chromatography.* A DEAE-Sepharose CL-6B column (2.5 × 30 cm) was equilibrated with buffer A containing 50 mM NH_4Cl. The pooled fractions of step 1 were diluted to 50 mM NH_4Cl and applied to the column. The bound material was eluted with a gradient from buffer A to buffer B containing 50 mM NH_4Cl and 50 mM KCl respectively. Fractions of 5 ml were collected and assayed for AMP incorporation (△----△) and poly(A) degradation by endonuclease (●——●) and exonuclease (○——○) as described in Experimental Procedures. Absorbance at 280 nm (——); pH gradient (-·-·-·-). Pooled fractions are indicated. Start of the elution with buffer C is indicated

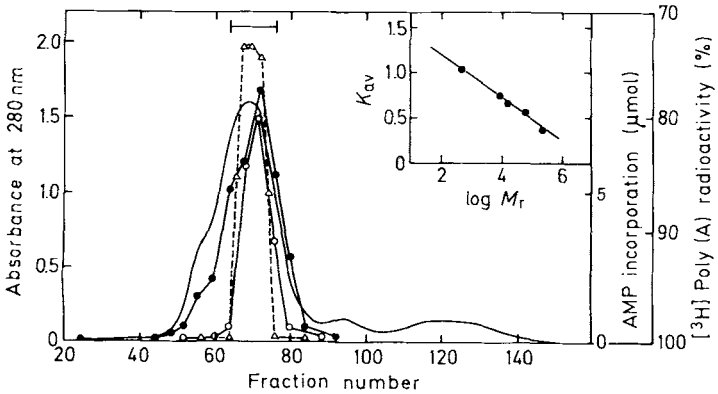

Fig. 3. *Gel filtration on CL-Sepharose 6B.* The column(5 × 80 cm) was equilibrated with buffer B containing 200 mM KCl. Fractions of 16 ml were collected at a rate of 80 ml/h and assayed for AMP incorporation (△----△), endonuclease (●——●) and exonuclease (○——○) activities as described in Experimental Procedures. Absorbance at 280 nm (——). Pooled fractions are indicated. Insert: calibration of the column with catalase (M_r 250000), bovine serum albumin (M_r 68000), chymotrypsin (M_r 25000); cytochrome *c* (M_r 12500) and ATP (M_r 507.22). Insert: K_{av} plotted as function of log M_r (●——●)

almost completely recovered in the poly(A)-polymerase-containing fractions. The specific activity of the poly(A) polymerase was increased 115-fold as compared to the specific activity of the enzyme in the postmitochondrial supernatant (Table 2).

Step 4. Ion-exchange chromatography on phosphocellulose P11. (a) Pooled fractions of Fig. 3 were chromatographed on a phosphocellulose P11 column equilibrated with buffer B containing 200 mM KCl. The AMP-incorporating activity was not bound in these conditions. However, the specific activity of the enzyme was further increased and the endonuclease activity was completely eliminated (Table 2). (b) After dilution of the flow of the phosphocellulose P11 column to a final concentration of 50 mM KCl, the AMP-incorporating activity was rebound to phosphocellulose P11 in buffer B containing 50 mM KCl. The elution was performed with a linear gradient from 50 mM to 200 mM KCl. The activity eluted between 100 mM and 160 mM KCl with an optimum at 135 mM KCl (Fig. 4). In this step only 7% of the applied protein was recovered in the active fractions concomitant with a loss of poly(A) polymerase activity of 75% (Table 2). This is due to a different kinetic behaviour of the enzyme before and after this chromatographic step. The

lag phase is donbled; 70% of the lost activity was recovered by doubling the assay incubation time. The specific activity of the enzyme was increased 510-fold. Only two proteins with M_r 94000 and 70000 were detected in the enzyme preparation by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (Fig. 5). The M_r-70000 protein was identified as poly(A) polymerase (see below).

Step 5. Affinity chromatography on poly(A)-Sepharose 4B and ATP-agarose. Contaminating poly(A)-binding proteins were removed by chromatography on poly(A)-Sepharose 4B. The pooled fractions of step 4 were dialysed against buffer D containing 50 mM KCl and applied to a poly(A)-Sepharose 4B column. Approximately 50% of the poly(A) polymerase activity was recovered in the unbound fraction. The poly(A) polymerase bound to the column was eluted with 750 mM KCl. Both activities have identical physical and enzymatic properties, e.g. M_r, ionic strength dependence, pH optimum, and only differ in primer specificity (our unpublished results). The unbound fraction of the poly(A)-Sepharose 4B column was further purified on ATP-agarose equilibrated with buffer D containing 50 mM KCl. The AMP-incorporating activity was recovered in the unbound fraction. The latter steps eliminated more than 60% of the applied proteins and all the exonuclease activity still contaminating the sample. The specific activity of poly(A) polymerase was almost doubled and was increased 950-fold as compared to the specific activity in the postmitochondrial supernatant (Table 2).

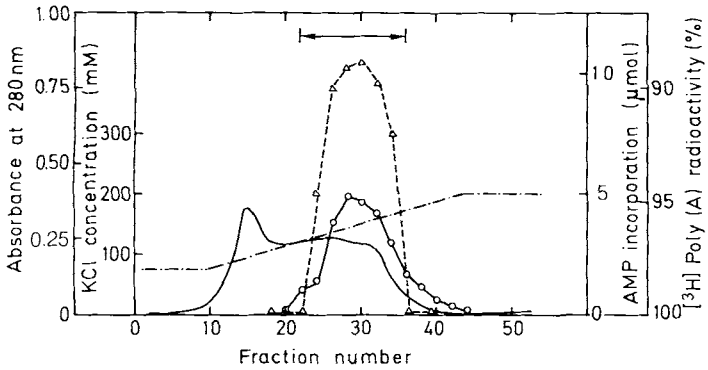

Fig. 4. *Ion-exchange chromatography on phosphocellulose P11.* Phosphocellulose P11 column (2 × 6 cm) was equilibrated with buffer B containing 50 mM KCl and loaded with the AMP-incorporating activity of step 4a diluted to 50 mM KCl with buffer B. The bound material was eluted with a salt gradient from 50 mM to 200 mM KCl in buffer B (–·–·–·–). Fractions of 3.5 ml were collected and assayed for AMP incorporation (△----△) and poly(A) degradation by exonuclease (○——○). Endonuclease activity was not detectable. Absorbance at 280 nm (——). Pooled fractions are indicated

Step 6. Concentration and storage of poly(A) polymerase. Poly(A) and ATP possibly leaked from the affinity columns were removed by chromatography on DEAE-cellulose equilibrated with buffer D containing 50 mM KCl. This step further increased the specific activity of the enzyme (Table 2). The filtrate was dialyzed overnight after addition of 2 ml DEAE-cellulose against 1 l of buffer E. After dialysis the supernatant was carefully removed and 2 vol. of buffer E containing 200 mM NH_4Cl added. After shaking for 10 min the sample was filtered and dialyzed against 1 l of buffer F for at least 4 h. This procedure resulted in a 30-fold concentration with a recovery of 72% of the poly(A) polymerase activity. After addition of ATP to a final concentration of 0.6 mM and incubation at 30°C for 30 min the enzyme was stored at −70°C. The stored enzyme remained active for at least two months.

Contaminating enzyme activities

Poly(A)-degrading activities were eliminated as shown in Table 2. Protease, tRNA:nucleotidyl transferase, poly-

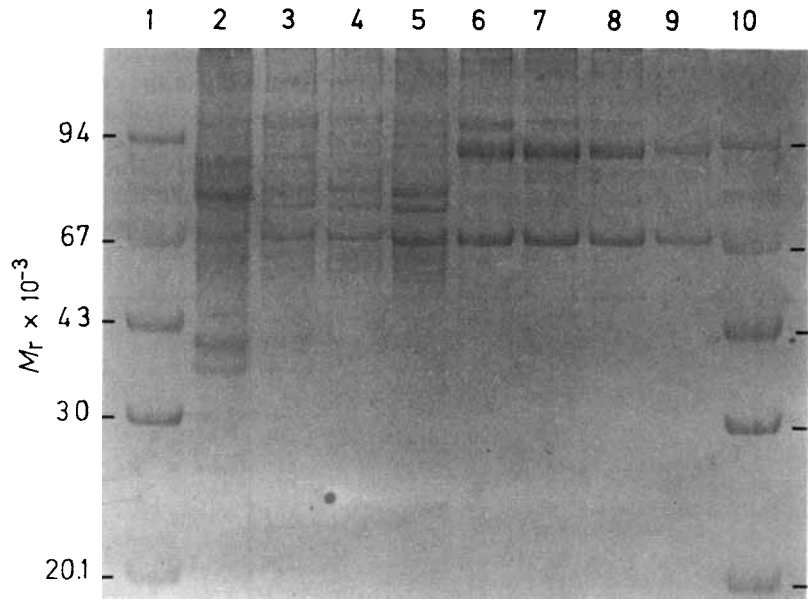

Fig. 5. *Sodium dodecyl sulphate gel electrophoresis of the purification steps.* Electrophoresis was on 10% polyacrylamide gels in the presence of 0.1% (w/v) sodium dodecyl sulphate as described in Methods. Marker proteins were phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14000). Lanes: (1) marker proteins; (2) DEAE-cellulose-1; (3) DEAE-Sepharose CL-6B; (4) CL-Sepharose 6B; (5) phosphocellulose P11 (a); (6) phosphocellulose P11 (b); (7) poly(A)-Sepharose 4B; (8) ATP-agarose; (9) DEAE-cellulose-2; (19) marker proteins

nucleotide phosphorylase, poly(ADP)-ribose polymerase, DNA-dependent RNA polymerase and protein kinase activities were not detected if assayed in the conditions described in Experimental Procedures.

Molecular mass of poly(A) polymerase

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the final enzyme preparation still revealed the presence of two proteins with M_r of 94000 and 70000 (Fig. 5). The M_r-70000 protein was identified as poly(A) polymerase by high-resolution liquid chromatography of the final preparation (Fig. 6). The poly(A) polymerase activity coincided with the protein eluted at an M_r of 68000 ± 2500. This value is in agreement with the results obtained by sucrose gradient centrifugation and gel filtration of the postmitochondrial supernatant. A sedimentation coefficient of 4.5 ± 0.2 S, equivalent to an M_r of 68000 – 73000, was calculated from centrifugation on a 5–20% (w/v) sucrose gradient in buffer A containing 200 mM NH_4Cl; an M_r of 70000 ± 5000 was calculated from gel filtration on a Sephacryl S-200 column equilibrated with buffer A containing 500 mM NH_4Cl (data not shown).

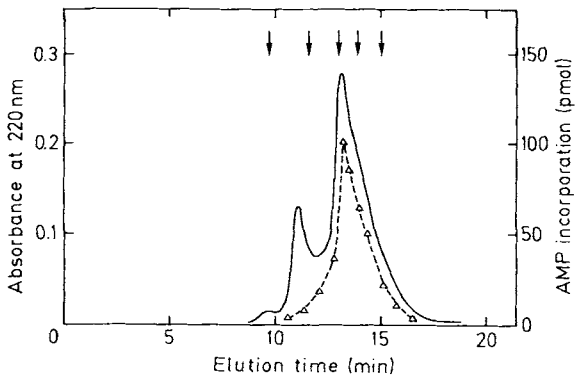

Fig. 6. *Analysis of poly(A) polymerase by high-resolution liquid chromatography.* The purified enzyme was dialyzed against buffer H and applied to an Ultro Pac TSK G 3000 SW column. Material was chromatographed at a constant rate of 640 µl/min. Fractions were collected and assayed for poly(A) polymerase activity (△----△). Absorbance at 220 nm (——). The column was calibrated with catalase (M_r 250000), lactate dehydrogenase (M_r 140000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000) and carbonic dehydrogenase (M_r 30000)

Characteristics of poly(A) polymerase

The enzyme is exclusively activated by Mn^+. In the presence of 0.45 mM ATP an optimum activity is obtained at a ratio of 1:2. Above 1 mM $MnCl_2$ the activity is decreased to a constant value (Fig. 7A). Addition of other divalent or monovalent ions inhibited the poly(A) polymerase activity. A decrease in enzyme activity of 50% is observed in the presence of 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $ZnCl_2$, 50 mM KCl or 50 mM NH_4Cl (Fig. 7B, C). The enzyme is active between pH 7.5 and pH 9.0 with an optimum at pH 8.4 [27]. Only ATP is used as a substrate by the enzyme and no oligonucleotide elongation is observed with CTP, GTP or UTP. However, competition between ATP and CTP, GTP or UTP is observed in mixing experiments. This effect may be explained by competition for the ATP-binding site of the enzyme, as indicated by the broadening of the lag phase in the initiation of polyadenylation [35].

As observed by others, oligo(A) synthesis did not proceed linearly as a function of time, enzyme concentration or primer concentration (for a review see [14, 15]).

DISCUSSION

In order to study the functional significance of polyadenylation of mRNP, an Mn^{2+}-activated poly(A) polymerase was purified to near homogeneity from the cytoplasm of cryptobiotic gastrulae of *Artemia salina*. Demonstration of the cytoplasmic origin of the enzyme is not trivial, due to the easy rupture of nuclei of these cryptobiotic embryos. After stabilization of fragile cell structures by Ficoll 400 [36], at least 80% of the nuclei remain intact during fractionation. The integrity of the nuclei was demonstrated by microscopy, measurement of DNA content, DNA-dependent RNA polymerase and poly(ADP)-ribose polymerase activities in nuclear, postnuclear and postmitochondrial fractions. The latter enzymes are believed to be nuclear enzymes [31, 37]. Measurement of the poly(A) polymerase activity in the different cellular fractions indicate that the $Mn^{2+} + (A)_4$-stimulated enzyme is mainly localized in the cytoplasm. Previously Sastre and Sebastian [18] localized 80% of the poly(A) polymerase activity in the cytoplasm and 20% in the nuclear and membrane fraction of *A. salina* using tRNA as a primer. The low amount of nuclear poly(A) polymerase is a consequence of cryptobiosis. Due to the absence of transcription, there is no need for nuclear polyadenylation. Approximately the same distribution is measured in unfertilized sea urchin

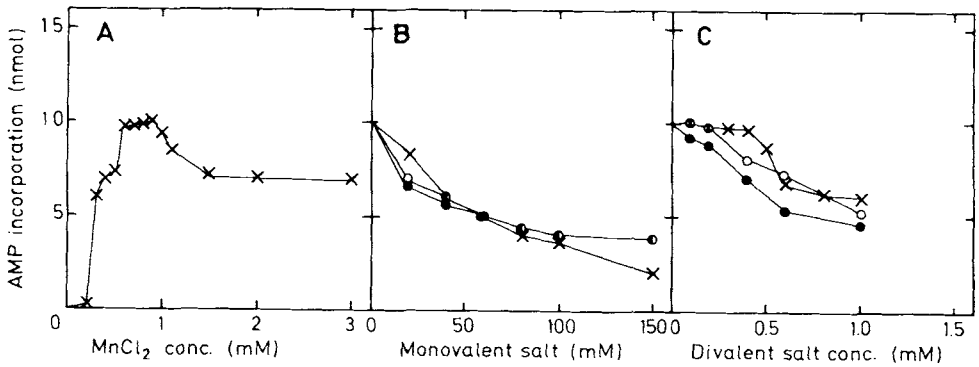

Fig. 7. *Effect of divalent and monovalent ions on poly(A) polymerase activity.* The assays were performed in standard conditions. The enzyme activity was measured as function of (A) $MnCl_2$, (B) NH_4Cl (×——×), KCl (○——○) and NaCl (●——●), (C) $MgCl_2$ (×——×), $ZnCl_2$ (○----○) and $CaCl_2$ (●——●). Assays in B and C were in the presence of 0.9 mM $MnCl_2$

eggs inactive in translation and in transcription [19]. It is also possible that the use of Mn^{2+} and $(A)_4$ results in a specific assay for cytoplasmic poly(A) polymerase. It has been shown that the cytoplasmic enzyme is activated by Mn^{2+} and oligo(A) primers [38, 39] in contrast to the nuclear enzyme which shows a preference for Mg^{2+} and RNA [38].

A purification procedure of the enzyme from the postmitochondrial supernatant has been developed. The main steps are (a) binding to DEAE-cellulose and DEAE-Sepharose CL-6B at a pH above 8.0; (b) gel filtration on CL-Sepharose 6B; (c) ion-exchange chromatography on phosphocellulose P11; (d) affinity chromatography on poly(A)-Sepharose 4B and ATP-agarose. The final preparation is free of other enzymatic activities and only contained two proteins with M_r of 94000 and 70000. The 70000-M_r protein was identified as poly(A) polymerase in agreement with the M_r value measured by Sastre and Sebastian [40] but is slightly higher than the M_r published for other organisms [38] in the presence of divalent ions.

In standard assay conditions the final preparation has a specific activity of 5.6 $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$.

The enzyme is exclusively activated by Mn^{2+}. The optimal Mn^{2+} concentration appears to be related to the ATP concentration. A relationship between divalent ion and substrate concentrations has also been observed for other organisms [15, 18]. The enzyme is very ionic-strength-sensitive. Low monovalent salt concentrations even inhibit the enzyme activity. In other systems poly(A) polymerase is stimulated at concentrations less than 50 mM KCl or NaCl (for a review see [15]). This activation is only observed with less purified poly(A) polymerase preparations of *A. salina* cryptobiotic gastrulae. The inhibition by Zn^{2+}, previously shown to activate nuclear poly(A) polymerase of rat liver [41], is important since this result confirms the cytoplasmic nature of the purified poly(A) polymerase.

In standard assay conditions an optimal enzyme activity is observed at pH 8.4. The same pH optimum has been measured by Tsiapalis et al. [39] and Sastre and Sebastian [40]. A pH optimum between 8 and 9 has been published for most poly(A) polymerases studied [15, 38].

The enzyme uses only ATP as substrate. This preference distinguishes the purified enzyme activity from enzymes using other nucleoside 5'-triphosphate. CTP, GTP and UTP inhibit the oligo(A) synthesis if included in the standard assay. The idea that the inhibition may be due to competition for ATP-binding sites of the enzyme is confirmed by the increase of the lag phase observed when AMP incorporation is studied as a function of time. The lag phase is believed to be a measure of the time needed to form the initiation complex [42–44] and is also observed when the enzyme activity is measured as a function of ATP, primer and enzyme concentration. If $(A)_4$ is used as primer, a decrease in reaction rate is observed at a total length of about 10 nucleotides. This phenomenon is influenced by the M_r-38000 poly(A)-binding and helix-destabilizing protein isolated from *A. salina* cryptobiotic embryos. Interaction of the synthesized oligo(A) primer with the poly(A)-binding protein resulted in a constant rate of polyadenylation [45].

The enzyme purified from *A. salina* cryptobiotic gastrulae has all the characteristics of poly(A) polymerase as reviewed by Rose and Jacob [15] and Edmonds [38]. These characteristics, in combination with the localization, identifies the enzyme activity as a cytoplasmic terminal riboadenylate transferase [39]. The enzyme preparation will be used to elucidate the function of poly(A) polymerase in the cytoplasm. Experiments concerning the primer specificity and polyadenylation of non-polysomal mRNP are in progress.

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