in the constants (within the experimental uncertainties) would improve the fit. Also at high NADH and enzyme concentrations, some nonspecific binding may occur.

The data in Figures 1 and 4 are in good agreement with those of Cassman and King (1972). However, the interpretation proposed here is more consistent with the direct binding data. The explanation for the different fluorescence enhancements is, of course, unknown but could be attributed to protein conformational changes or to structurally nonequivalent sites.

The binding of NADH to porcine *s*-malate dehydrogenase appears to involve two equivalent sites on the dimer (Holbrook and Wolfe, 1972) as in the model proposed here for the beef heart enzyme, with the binding constants being somewhat larger for the porcine enzyme. The temperature-jump data provide indirect evidence for a simple binding mechanism, such as equivalent sites, since only a single relaxation process is observed under conditions where the enzyme is predominantly dimer. A more complex relaxation spectrum would be expected for cooperative binding. The second-order rate constant estimated from the data is close to the maximum possible rate constant for an enzyme–substrate reaction (Alberty and Hammes, 1958).

In summary, the binding of NADH to beef heart *s*-malate dehydrogenase appears to involve nearly equivalent or equivalent enzyme binding sites in both monomer and dimer, with the fluorescence enhancement of NADH depending on the type of site occupied.

Acknowledgment

The authors are indebted to Professor S. Edelstein for several illuminating discussions and to Miss Jean Hsu for her technical assistance.

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Mitochondrial Poly(A) Polymerase from a Poorly Differentiated Hepatoma: Purification and Characteristics†

Kathleen M. Rose,* Harold P. Morris,‡ and Samson T. Jacob*

ABSTRACT: Poly(A) polymerase (EC 2.7.7.19) solubilized from mitochondria of a poorly differentiated rat tumor, Morris hepatoma 3924A, was purified more than 1000-fold by successive column chromatography on phosphocellulose, DEAE-Sephadex, and hydroxylapatite. Purified enzyme catalyzed the incorporation of ATP into poly(A) only upon addition of an exogenous primer. Of several primers tested, synthetic poly(A) was the most effective. The enzyme utilized mitochondrial RNA as a primer at least five times as efficiently as nuclear RNA. The enzyme required Mn^{2+} and had a pH optimum of 7.8–8.2. The enzyme utilized ATP exclusively as a substrate; the calculated K_m for ATP was 28 μM. The polymerization reaction was not inhibited by RNase, ethidium bromide, distamycin, or α-amanitin. The reaction was sensitive to *O-n*-octyloxime of 3-formylrifamycin SV (AF/013). As estimated from glycerol gradient centrifugation and acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the molecular weight of the enzyme was 60,000. The product was covalently linked to the polynucleotide primer and the average length of the poly(A) formed was 600 nucleotides.

Poly(A) polymerase, the enzyme that catalyzes the incorporation of adenylic acid residues into poly(A), is widely distributed in nature. Thus, the enzyme is present in a variety of bacteria (August et al., 1962; Gottesman et al., 1962; Hardy and Kurland, 1966; Payne and Boezi, 1970; Colvill and Terzi, 1968; Ohasa and Tsugita, 1972; Modak and Sri-

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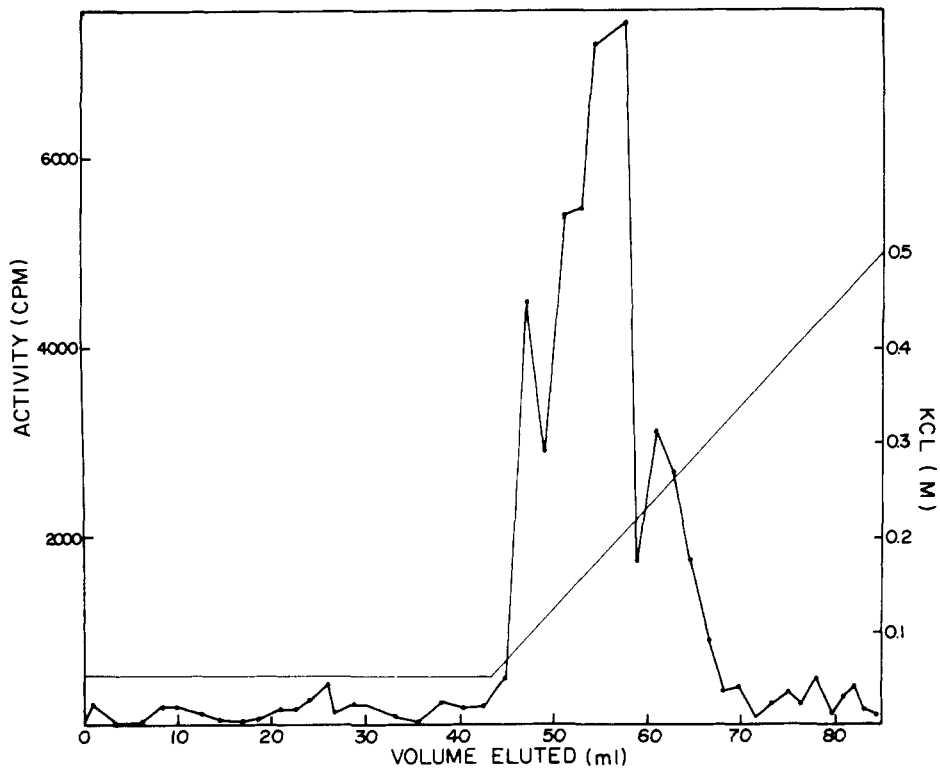

FIGURE 1: Hydroxylapatite column chromatographic profile of mitochondrial poly(A) polymerase from hepatoma 3924A. The partially purified enzyme containing 20 mg of protein was layered on 2.4 × 5 cm hydroxylapatite (Bio-Rad) column equilibrated with PGMED (pH 6.8) containing 0.05 *M* KCl and washed with 10 ml of the same buffer. The enzyme activity was eluted with a linear gradient of 0.05–0.8 *M* KCl in PGMED buffer. Aliquots (0.1 ml) were assayed for poly(A) polymerase activity as described in the text.

nivasan, 1973), lower eukaryotes (Biswas et al., 1965; Sachar, 1968; Mans, 1971; Twu and Bretthauer, 1971; Haff and Keller, 1973), and in higher organisms (Burdon, 1963; Kato and Kurokawa, 1970; David et al., 1971; Winters and Edmonds, 1973; Niessing and Sekeris, 1973; Tsiapalis et al., 1973). The eukaryotic enzyme has been found in the nuclear, ribosomal, and post-microsomal fractions of the cell, and, recently, Jacob and Schindler (1972a) solubilized a similar enzyme from rat liver mitochondria. Subsequent studies (Jacob and Schindler, 1972b) showed that the activity of the mitochondrial enzyme was relatively low in a series of poorly differentiated Morris hepatomas.

Since a post-transcriptional addition of a poly(A) segment consisting of 150–250 adenylic residues in the 3′ position appears to be a prerequisite for the proper processing of nuclear mRNA in eukaryotic cells (Darnell et al., 1971; Philipson et al., 1971; Darnell et al., 1973), the enzyme that synthesizes this ubiquitous homopolymer has received considerable attention. Recent studies (Ojala and Attardi, 1974a,b) clearly demonstrating the existence of poly(A) in the 3′ position of HeLa cell mitochondrial mRNA imply a functional role for the mitochondrial poly(A) polymerase in extranuclear mRNA metabolism. To elucidate further the role of mitochondrial poly(A) polymerase in normal cells and to investigate the cause for the relative lack of enzyme activity in the hepatomas, we purified the enzyme extensively from normal rat liver and hepatomas. This paper describes the purification and properties of poly(A) polymerase from the mitochondria of a poorly differentiated, rapidly growing tumor, Morris hepatoma 3924A.

Experimental Procedure

Isolation of Mitochondria. Hepatoma-bearing rats were sacrificed 28 days after implantation of hepatoma 3924A (doubling time = 4.4 days). Tumors were excised, freed of necrotic tissues, and suspended in 0.9% NaCl–0.25 *M* sucrose. Each hepatoma yielded 7–15 g of tissue. The tumors were minced and homogenized (2 strokes with a Teflon–glass homogenizer) with 5 volumes of sterile Tris buffer (pH 7.4) containing 0.25 *M* sucrose and 1 m*M* EDTA.

The homogenate was filtered and centrifuged for 10 min at 1000*g*. The supernatant was recentrifuged at the same speed to completely remove the nuclei. The supernatant was then centrifuged at 8000*g* for 10 min to sediment the mitochondria. The pellet was suspended in 1 ml of the Tris buffer/g of hepatoma and recentrifuged at 8000*g* for 10 min. The mitochondrial pellet was then suspended (1 ml/12 g of tissue) in 50 m*M* Tris (pH 9.0) containing 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol, and 20 m*M* KCl (TMEDK buffer). In some experiments mitochondria were further purified by digitonin treatment to remove adhering lysosomes and cytoplasmic ribosomes (Schnaitman and Greenawalt, 1968; Lowenstein *et al.*, 1970; Malkin, 1971). The specific activity of enzyme extracted from digitonin treated mitochondria was the same as the enzyme extracted without digitonin treatment.

Solubilization of Poly(A) Polymerase. The enzyme was solubilized from the isolated mitochondria by sonication (3 × 15 sec) in TMEDK (pH 9.0), addition of glycerol to 20% (v/v), and incubation at 37° for 30 min as described previously (Jacob and Schindler, 1972a). The extract was then centrifuged at 105,000*g* for 75 min. The supernatant contained the poly(A) polymerase activity.

Purification of Poly(A) Polymerase. The solubilized enzyme was dialyzed against 50 m*M* potassium phosphate buffer (pH 6.8) containing 20% glycerol (v/v), 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol, and 50 m*M* KCl (PGMEDK buffer) and purified by phosphocellulose

Table I: Purification of Poly(A) Polymerase from Mitochondria of Morris Hepatoma 3924A.

Fraction	Protein (mg)	Specific Activity (units/mg)	Total Units
A. Mitochondrial lysate (400 g of tissue)	440	0.03	13
B. Solubilized extract (supernatant)	212	0.15	32
C. Phosphocellulose column	42	3.0	126
D. DEAE-Sephadex column	25	6.2	155
E. Hydroxylapatite column	6	40	240

chromatography. The enzyme, which eluted at 0.25 *M* KCl, was dialyzed against TGMEDK (pH 8.2, 50 m*M* potassium phosphate replaced by Tris) and rechromatographed on a DEAE-Sephadex column as described previously (Jacob et al., 1974a). The 0.05 *M* KCl wash fractions of the DEAE-Sephadex column, containing the enzyme activity, were pooled, concentrated approximately twofold with dry Sephadex G-25 (coarse), and dialyzed overnight against PGMEDK. The dialyzed fraction containing 15–20 mg of protein was layered on a hydroxylapatite column (2.4 × 5 cm) which had been equilibrated in PGMEDK. After washing with 10 ml of the same buffer, a linear gradient (0.05–0.8 *M* KCl in PGMED) was applied; 1-ml fractions were collected and 0.1 ml was taken to assay for enzymatic activity (Figure 1). The fractions containing the peak activity were pooled and dialyzed for several hours against 50 m*M* Tris (pH 7.8) containing 50% glycerol (v/v). This dialysis concentrated the enzyme twofold and the final protein concentration was 200–300 μg/ml as determined by the method of Lowry et al. (1951). The enzyme was stored at −90°.

Assay for Poly(A) Polymerase Activity. The enzymatic activity was measured in a reaction mixture containing 33 m*M* Tris-HCl (pH 8), 0.5 m*M* $MnCl_2$, 25 m*M* KCl, 240 μ*M* [2,8-^{3}H]ATP (New England Nuclear, 5 × 10^3 cpm/nmol), 120 μg of Poly(A) (Miles Laboratories), and 10–20 μg of enzyme protein in 0.3 ml for 60 min at 37°. The reaction was stopped by the addition of 1 ml of 10% Cl_3CCOOH, containing 0.04 *M* $Na_4P_2O_7$, filtered, washed, and counted in a liquid scintillation counter as described previously (Jacob and Schindler, 1972a). One unit of enzyme is that which incorporated 1 nmol of AMP into acid-insoluble material in 60 min.

Glycerol Gradient Centrifugation. The purified enzyme (10–20 μg) was layered on a 10–30% glycerol gradient (4.5 ml) containing 50 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 0.1 m*M* dithiothreitol, and 0.1 m*M* EDTA. The sample and appropriate standards were run on parallel gradients in the SW56 rotor for 15 hr at 42,000 rpm.

Acrylamide Gel Electrophoresis. Disc electrophoresis on 5% cross-linked polyacrylamide gels was carried out as described by Shapiro et al. (1967) utilizing a sodium dodecyl sulfate–sodium phosphate (pH 7.1) buffer. Protein samples were treated for 4 hr at 37° in 0.1 *M* sodium phosphate (pH 7.1), 1% sodium dodecyl sulfate, and 1 m*M* dithiothreitol. The samples were then dialyzed overnight against a 10% (v/v) solution of the above buffer; 5–20 μg of enzyme or protein standard was applied to the gel and current was applied at 7.5 mA/gel until the tracking dye (Bromophenol Blue) reached the bottom of the gels (approximately 2 hr). Gels were stained 3 hr with Coomaasie Brilliant Blue (0.25% in MeOH–HOAc–H_2O, 5:1:5, v/v) and destained by several days of equilibration in the same solution without stain. The absorbance of the protein at 550 nm was then determined using a Gilford 2400 spectrophotometer equipped with a gel scanning attachment.

Table II: Effect of Various Polynucleotides on Poly(A) Polymerase from Hepatoma 3924A.[a]

Addition	Concentration (μg/ml)	nmoles of AMP per mg per hr
Poly(A)	360	40.0
Mitochondrial RNA (rat liver)	300	8.0
Mitochondrial RNA (hepatoma 3924A)	300	10.6
Nuclear RNA (rat liver)	300	2.3
tRNA (*E. coli*)	450	4.3
$(A_p)_3A$	360	3.8
Poly[d(A-T)]	300	1.5
Poly(U)	600	1.5
DNA (calf thymus)	450	0
Denatured DNA (calf thymus)	450	0.6
H_2O		1.5

[a] The standard assay mixture contained 20 μg of purified enzyme and the additions as indicated in the table. The assays were performed in triplicate. The results are the average of three separate experiments.

Results

Purification. The purification steps are summarized in Table I. The specific activity of the purified enzyme ranged from 30 to 50 nmol of AMP incorporated per mg per hr. This represents a purification of more than 1000-fold. The total enzyme units increased almost threefold upon solubilization, probably due to removal of a poly(A) degrading nuclease (Jacob and Schindler, 1972a). The increase in total activity of the enzyme upon chromatography, as assayed with saturating levels of exogenous primers, is largely due to removal of inefficient primers from the enzyme (Jacob et al., 1974a). After hydroxylapatite chromatography (Figure 1), the enzyme was stable in 50% glycerol for 4 weeks at −90° without appreciable loss of activity and for 5 months with about 40% reduction in activity. The final preparation was devoid of poly(A) degrading nuclease and RNase.

Metal Ion, pH, and Ionic Strength Optima. The enzyme was more active with Mn^{2+} than with Mg^{2+}. The enzyme had a Mn^{2+} optima ranging from 0.25 to 1.0 m*M*. The Mn^{2+}/Mg^{2+} activity ratio at optimal Mn^{2+} and Mg^{2+} concentrations was approximately 3.5. The KCl concentration which gave maximal activity was 33 m*M*. The reaction was inhibited at KCl concentrations greater than 150 m*M*. When NaCl was substituted for KCl at 33 m*M*, the activity was reduced by 66%. The enzyme was optimally active at pH 7.8–8.2.

Primer Requirements. The solubilized enzyme did not

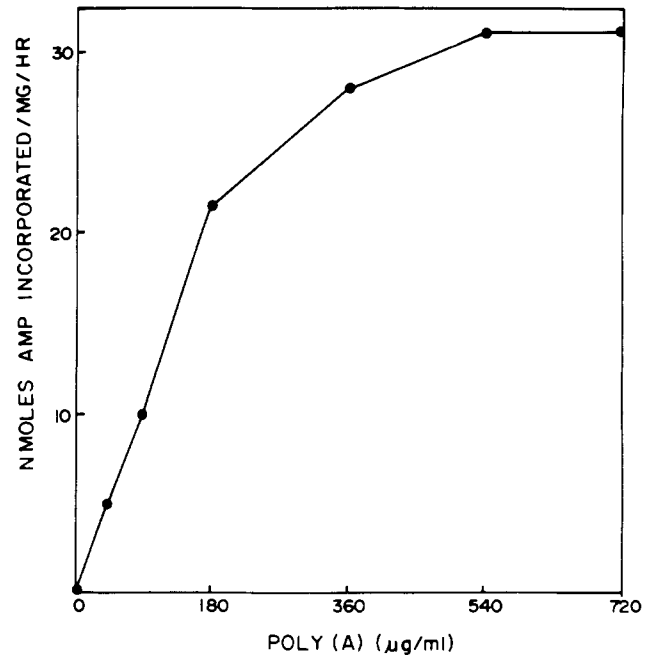

FIGURE 2: Effect of poly(A) concentration on hepatoma mitochondrial poly(A) polymerase. Reaction mixtures containing 15 μg of enzyme were prepared as described in the text except that the poly(A) concentration was varied as indicated.

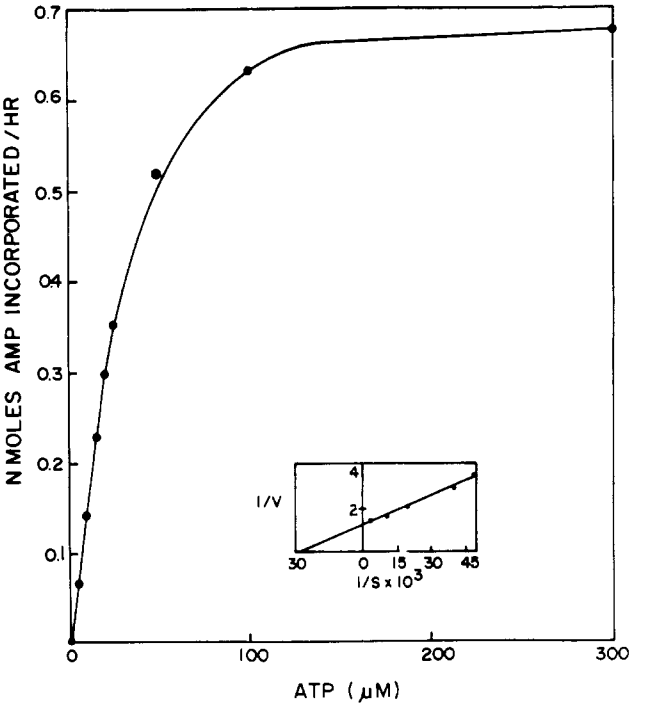

FIGURE 3: Effect of ATP concentration on the hepatoma mitochondrial poly(A) polymerase activity. Reaction mixtures containing 12 μg of enzyme were prepared as described in the text except that the concentration of ATP was varied as indicated. Insert shows a double reciprocal plot of $1/V$ *vs.* $1/S$.

respond to addition of primer. However, purification of the enzyme on phosphocellulose resulted in its dependence upon exogenous primer. Either synthetic poly(A) or a primer obtained from solubilized liver poly(A) polymerase (molecular weight <30,000) could be utilized (Jacob et al., 1974a). Any unprimed reaction carried out by the purified enzyme was never more than 5% of the primer-dependent reaction. Table II shows the effect of various polynucleotide primers

Table III: Substrate Specificity of 3924A Poly(A) Polymerase.[a]

Substrate ^{3}H	Additions	Incorporation (nmol per mg per hr)
ATP		80
ATP	UTP	67
ATP	CTP	68
ATP	GTP	46
ATP	UTP, CTP, GTP	24
UTP	ATP, CTP, GTP	0.2
CTP	ATP, UTP, GTP	0
GTP	ATP, CTP, UTP	0.08
ADP		4
dATP		6

[a] Assay mixtures containing 10 μg of enzyme were incubated with 120 μg of poly(A) in the standard reaction mixture with the additions as indicated. The specific activities of the nucleotide substrates (New England Nuclear) were 10,000–20,000 cpm/nmol.

on 3924A poly(A) polymerase activity. The purified enzyme was active only when commercial poly(A) or total mitochondrial RNA was used as the primer. Due to technical difficulty in obtaining sufficient quantities of mitochondrial RNA, especially from hepatomas, for use as primer, poly(A) itself was used for routine experiments. Poly[d(A-T)], DNA, nuclear RNA, or tRNA were not efficient primers. The effect of varying amounts of poly(A) upon the enzyme activity is illustrated in Figure 2. The activity of the enzyme increased linearly with increasing poly(A) concentrations and reached a plateau at 540 μg/ml (approximately 5 μ*M*).

Substrate Specificity. Table III shows the substrate specificity of the reaction. Purified enzyme utilized ATP almost exclusively. Addition of UTP or CTP to the reaction mixture resulted in a 15% inhibition of ATP incorporation and increased inhibition was observed with GTP. The inhibition by UTP, CTP, and GTP was somewhat additive, i.e., when all were present in the reaction mixture, ATP incorporation was reduced by 76%. No nucleotide triphosphate other than ATP was significantly incorporated into product either in presence or absence of other nucleoside triphosphates. The enzyme exhibited typical Michaelis-Menten kinetics with increasing ATP concentrations. ATP reached saturating levels at 100 μ*M* (Figure 3). The K_m for ATP as determined from a Lineweaver-Burk plot was 28 μ*M* at enzyme levels that gave a linear relationship of the reaction velocity to enzyme concentrations (Figure 3, insert).

Kinetics of the Reaction. The reaction was linear for almost 90 min at 37° (Figure 4). No initial lag in the reaction could be detected and no decline in the enzymatic activity in 210 min was observed. Figure 5 shows the rate of the reaction plotted against enzyme concentration. At low enzyme levels the rate is not directly proportional to the enzyme concentration. Addition of bovine serum albumin (1 mg/ml) to the reaction mixture to stabilize the protein had no effect on the rate of the reaction. Reactions run in the absence of KCl or in the presence of a higher (75 m*M*) KCl concentration produced curves which were superimposable to that shown in Figure 5. Since poly(A) functions both as primer and product, it is possible that the nonlinear rela-

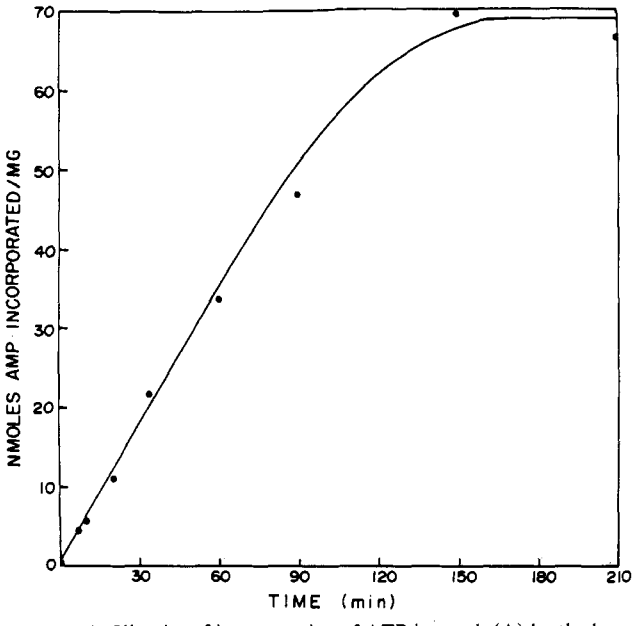

FIGURE 4: Kinetics of incorporation of ATP into poly(A) by the hepatoma mitochondrial poly(A) polymerase. The enzyme reaction containing 20 μg of enzyme was terminated at the times indicated.

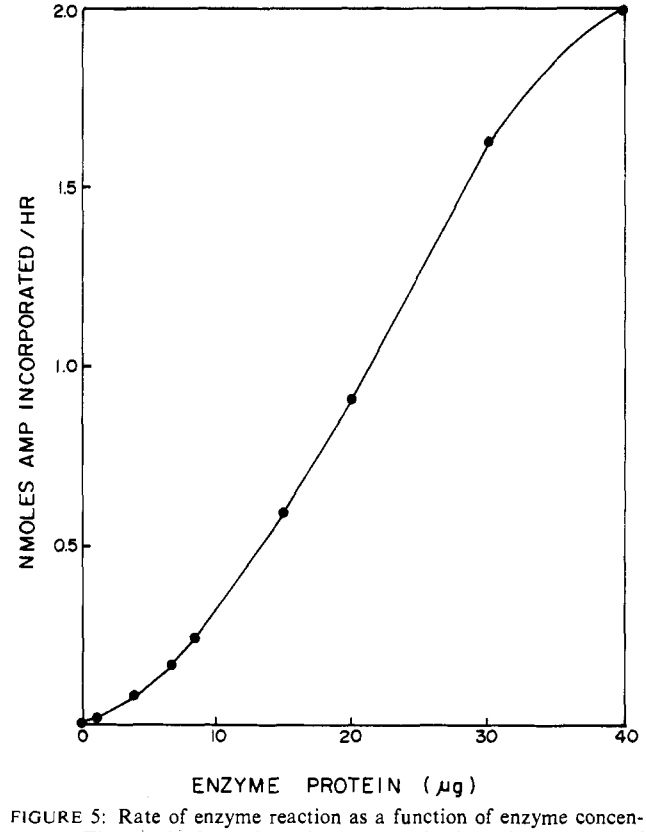

FIGURE 5: Rate of enzyme reaction as a function of enzyme concentration. The standard reaction mixtures contained varying amounts of enzymatic protein as indicated.

tionship of reaction velocity to enzyme concentrations at low enzyme levels may be a result of product inhibition.

Effect of Inhibitors on 3924A Poly(A) Polymerase Activity. Table IV shows the effect of various inhibitors on the reaction. The enzyme was not inhibited by *N*-ethylmaleimide. Preliminary experiments showed an inhibition by another sulfhydryl binding agent, *p*-chloromercuribenzoate. However, this inhibition was not reversible by addition of excess dithiothreitol and was presumably a nonspecific inhibition of the enzyme. Potassium phosphate did not inhibit the reaction; however, sodium pyrophosphate completely abolished product formation. Inhibitors of template dependent reactions (distamycin, ethidium bromide, and actinomycin D) also had no effect on the product formation. Similarly, inclusion of DNase or RNase in the reaction mixture did not reduce the amount of poly(A) product formed. Neither streptolydigin, a specific inhibitor of elongation of bacterial RNA polymerase (Giovanni et al. 1971), nor α-amanitin, an inhibitor of elongation of mammalian RNA polymerase II (Jacob et al., 1970; Jacob, 1973), inhibited the poly(A) polymerase activity. Indeed, the only compound found to inhibit the enzyme was the rifamycin derivative AF/013 (*O-n*-octyloxime of 3-formylrifamycin SV). This drug has recently been shown to inhibit mitochondrial poly(A) polymerase from rat liver by competing with the substrate (Jacob et al., 1974b).

Table IV: Effect of Various Inhibitors on Poly(A) Polymerase from Hepatoma 3924A.[a]

Addition	Concentration	% Activity
None		100
N-Ethylmaleimide	2×10^{-5} *M*	100
	2×10^{-3} *M*	100
Distamycin	180 μg/ml	100
Ethidium bromide	100 μg/ml	100
Actinomycin D	50 μg/ml	100
DNase	60 μg/ml	100
RNase	10 μg/ml	95
α-Amanitin	1.5 μg/ml	100
	7.5 μg/ml	100
Streptolydigin	3 μg/ml	95
	30 μg/ml	95
	300 μg/ml	95
AF/013	75 μg/ml	30
	150 μg/ml	0
Potassium phosphate (pH 8)	10 m*M*	120
Sodium pyrophosphate	10 m*M*	0

[a] 15 μg of purified enzyme was assayed with the standard reaction mixture containing 360 μg/ml of poly(A). 100% activity corresponds to 0.8 nmol of AMP incorporated/hr.

Molecular Weight. The sedimentation constant of the enzyme was found to be 4.2 S by glycerol gradient centrifugation (Figure 6). On the assumption of globular conformation, this represents a molecular weight of 60,000. It should be noted that higher molecular weights and a pattern of enzyme aggregation were obtained when the enzyme was either centrifuged in lower ionic media or not allowed to fully equilibrate with KCl before application to the gradient. Analysis of the enzyme by acrylamide gel electrophoresis in the Tris-glycine (pH 9) system as adapted by Davis (1964) also indicated enzyme aggregates (molecular weights ranging from 100,000 to 500,000). Electrophoresis in the presence of sodium dodecyl sulfate (Shapiro et al., 1967) indicated that at least 75% of the protein migrated with a calculated molecular weight of 60,000 (Figure 7), agreeing with the results from glycerol gradient analysis. A minor protein peak migrated on SDS acrylamide gels with a calculated molecular weight of 120,000. Whether this represents an enzyme dimer resistant to SDS is not known. Assuming a

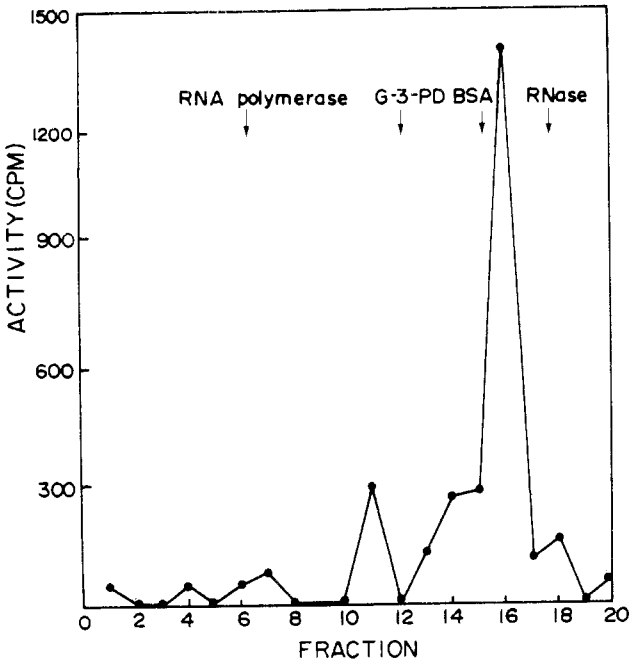

FIGURE 6: Glycerol gradient sedimentation of the hepatoma mitochondrial poly(A) polymerase. The purified enzyme (20 μg) was diluted sixfold with 50 m*M* Tris-HCl (pH 8.0) containing 100 m*M* KCl, 100 m*M* dithiothreitol, 100 m*M* EDTA, and 3% glycerol. The enzyme was layered on a 10–30% glycerol gradient (4.5 ml) in the same buffer. Four gradients containing 500 μg each of bovine serum albumin (BSA, 4.5 S), ribonuclease A (RNase, 2.0 S), glyceraldehyde-3-phosphate dehydrogenase (G-3-PD, 7.0 S), or 2 units of *E. coli* K12 RNA polymerase (15 S, Miles Laboratories) were run in parallel as mentioned. The gradients were centrifuged for 15 hr at 4°, at 42,000 rpm in an SW 56 rotor. Fractions of 0.175 ml were collected and each fraction was analyzed as follows: hepatoma poly(A) polymerase as described in the text; RNA polymerase as described by Jacob et al. (1970), BSA, glyceraldehyde-3-phosphate dehydrogenase, and RNase for protein as described by Lowry et al. (1951).

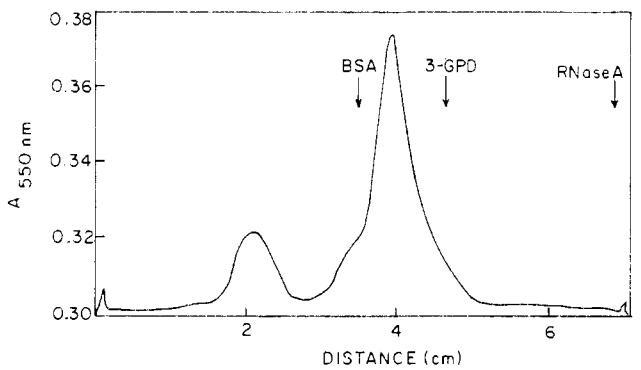

FIGURE 7: Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Purified enzyme was treated with sodium dodecyl sulfate and dithiothreitol as described in the text; 8–10 μg of enzyme or appropriate standard were applied to 5% cross-linked gels (Shapiro et al., 1967) and current (7.5 mA/gel) was applied for 2 hr. Gels were stained and destained, and absorbance was determined using a Gilford 2400 spectrophotometer equipped with a gel scanning attachment.

molecular weight of 60,000, the enzyme is optimally active when the molar ratio, enzyme:poly(A):ATP:Mn^{2+} approximately equals 1:10:200:200.

Product of the Reaction. The product was shown to be poly(A) by its insensitivity to RNase, its retention on Millipore filters in the presence of 0.5 *M* KCl, and its binding to poly(U)-impregnated filters. The product formed in a 60-min reaction mixture performed as described under Experimental Procedures, substituting [^{14}C]ATP for [^{3}H]ATP,

Table V: Sequential Degradation of Poly(A) by Venom Phosphodiesterase.[a]

Time (min)	^{3}H (cpm)	^{14}C (cpm)	^{3}H/^{14}C
0	1730	38	45.5
10	901	38	23.8
20	467	29	16.1
30	116	30	3.9
40	52	15	3.5
60	0	0	0

[a] Commercial poly(A) was incubated in the standard reaction mixture with 15 μg of enzyme, replacing [^{3}H]ATP with [^{14}C]ATP for 10 min. The [^{14}C]poly(A) was then precipitated with 2.5 volumes of 98% ethanol containing 2% potassium acetate and kept at −20° overnight. The poly(A) was centrifuged, dissolved in a small volume of water, and recentrifuged to remove undissolved protein. The [^{14}C]-poly(A) was then used as a primer for a standard reaction mixture using [^{3}H]ATP (1 hr). The final product was then precipitated with ethanol as above; 5–10 A_{260} units of the [^{3}H-^{14}C]poly(A) were incubated at 37° in a 2-ml reaction mixture containing 10 m*M* Tris (pH 7.9), 5 m*M* $MgCl_2$, and 8 units of viper venom phosphodiesterase (Calbiochem); 0.3-ml aliquots were removed at the times indicated and precipitated with 2 ml of 10% Cl_3CCOOH containing 0.04 *M* $Na_4P_2O_7$ and filtered as described in the text. The assay was performed in triplicate. The cpm expressed were corrected for background and channel overlap.

was subjected to 0.3 *N* KOH hydrolysis for 18 hr, neutralized, and then chromatographed on PEI paper according to the procedure of Randerath and Randerath (1967); 95,000 cpm was obtained in the AMP spot; 150 cpm remained with adenosine, accounting for almost 98% recovery of radioactivity originally present. No radioactivity was detected anywhere else on the chromatograph. The ratio of internal AMP to adenosine indicated an average chain length of more than 600 nucleotides.

Covalent Attachment of the Product to the 3′ Terminus of the Poly(A) Primer. Poly(A) was used as a primer for a short time (10 min) labeling of the product with [^{14}C]AMP as described in the legend to Table V. The [^{14}C]poly(A) product was then used as the primer for a 1-hr enzymatic reaction. The [^{14}C-^{3}H]poly(A) formed was then subjected to viper venom phosphodiesterase degradation. Since this enzyme is an exonuclease specific for the 3′ terminus, reduction in the ^{3}H cpm before reduction in ^{14}C cpm would show (a) the poly(A) polymerase reaction product is covalently linked to its primer and (b) the polymerization reaction occurs in the 5′ to 3′ direction. As shown in Table V, the ratio ^{3}H/^{14}C decreased with time.

An analogous experiment (Table VI) was carried out using spleen phosphodiesterase, an exonuclease specific for the dephosphorylated 5′ terminus. The substrate for the spleen enzyme was poly(A) labeled in reversed order (a short incubation with [^{3}H]ATP followed by incubation with [^{14}C]ATP). After removal of the 5′ phosphate with alkaline phosphatase, the time course of degradation of the labeled poly(A) by spleen phosphodiesterase showed a sequential degradation in the 5′ to 3′ direction, thus supporting the results with venom diesterase.

The covalent attachment of the product to primer was further confirmed by measuring the incorporation of [γ-^{32}P]ATP into poly(A). Substituting [γ-^{32}P]ATP (30,000

Table VI: Sequential Degradation of Poly(A) by Spleen Phosphodiesterase.[a]

Time (min)	^3H (cpm)	^{14}C (cpm)	$^3H/^{14}C$
0	335	1236	0.271
10	290	1235	0.235
20	175	925	0.189
40	115	725	0.158
70	25	225	0.111
90	0	75	0

[a] Commercial poly(A) was incubated in the standard reaction mixture with 15 μg of enzyme using [^{3}H]ATP for 10 min. The [^{3}H]poly(A) was then precipitated with ethanol as described in the legend to Table V. The labeled poly(A) was then used as a primer for a new reaction mixture containing [^{14}C]ATP for 1 hr. The final product was precipitated with ethanol as described previously. The radioactive poly(A) was dephosphorylated with alkaline phosphatase; 5–10 A_{260} units of poly(A) was incubated in a 1-ml reaction mixture containing 10 m*M* Tris-HCl (pH 8.3) and 20 μg of alkaline phosphatase for 4 hr at 37°. Alkaline phosphatase was denatured by heating the reaction mixture at 90° for 2 min. The poly(A) was precipitated with ethanol as described previously; 3–4 A_{260} units of dephosphorylated [^{14}C-^{3}H]poly(A) was then incubated in a 1.4-ml reaction mixture containing 100 m*M* potassium phosphate (pH 6.8) and 1.0 unit of spleen phosphodiesterase (Boehringer); 0.2-ml aliquots were removed at the times indicated, precipitated with Cl_3CCOOH, and filtered as described in the text. The assay was performed in triplicate and the cpm expressed were corrected for background and channel overlap.

cpm/nmol) for [^{3}H]ATP in the standard reaction mixture, no incorporation of γ-^{32}P was observed, suggesting the absence of 5′-P termini in the reaction product. However, under similar conditions, the enzyme incorporated 0.6 nmol of [^{3}H]ATP/hr into poly(A). Thus, there was no detectable initiation of poly(A) chains by the hepatoma poly(A) polymerase and the product of the reaction was therefore attached to the 3′-OH terminus of the poly(A) primer. When the incorporation of [γ-^{32}P]ATP into RNA was simultaneously measured in a template dependent reaction using purified RNA polymerase II from rat liver (Jacob, 1973), about 0.5% of the total AMP units incorporated was at the 5′-P terminus showing initiation of RNA chains.

Discussion

The present studies describe the purification and characterization of mitochondrial poly(A) polymerase solubilized from a poorly differentiated tumor, Morris hepatoma 3924A. Jacob and Schindler (1972b) have previously reported that prior to purification the activity of the mitochondrial poly(A) polymerase in at least three poorly differentiated hepatomas is only a small fraction (2–10%) of the activity of the corresponding liver enzyme. The relatively low activity of the solubilized tumor mitochondrial poly(A) polymerase (Jacob and Schindler, 1972b) is partially due to a reduced level of the enzyme (total units of purified enzyme per gram of tissue or per milligram of mitochondrial protein) which results from reduced size and/or number of hepatoma mitochondria (Allard et al., 1952; Hruban et al., 1972), but it is largely due to the presence of inefficient primer(s) associated with the enzyme (Jacob et al., 1974a).

It should be pointed out that a reduction in the activity of other mitochondrial enzymes (assayed in the crude state) including adenylate kinase, monoamine oxidase, cytochrome oxidase, and other enzymes (Pedersen et al., 1970; Criss, 1971; Schriber et al., 1970; White et al., 1974) has been reported in several fast-growing hepatomas including Morris hepatomas. The most extreme case is the virtual absence of monoamine oxidase in the mitochondria from melanoma cells (White et al., 1974). The same authors have concluded that the biochemical and structural aberrations observed in the Novikoff hepatoma are an inherent property of the neoplastic cells and not related to growth conditions. A reduction in adenylate kinase could be responsible for significantly lower levels of ATP reported (Mintz et al., 1967) in rapidly proliferating tumors. The reduced levels of ATP could then result in decreased in vivo synthesis of poly(A) in some tumors.

One of the most striking features of the purified 3924A enzyme is its capacity to add as much as 600 nucleotides to preexisting poly(A) primer. This is probably the highest number of adenylic acid units added to a primer by any poly(A) polymerase. Several factors such as primer and substrate concentrations, time of incubation, etc., are known to affect the chain length of the product formed in vitro. It is possible that the conditions used in our experiments are optimal for the production of a long poly(A) chain. The length of the poly(A) formed in vivo by the tumor enzyme could be controlled by several factors. One such factor could be a potent poly(A)-degrading nuclease identified in liver mitochondria (Jacob and Schindler, 1972a), which may be similar to polynucleotide phosphorylase localized in the mitochondria (See and Fitt, 1972).

Unlike the primer-dependent poly(A) polymerase of bacteria, the functional significance of nuclear poly(A) polymerase seems clear, the latter being the most likely enzyme to add poly(A) to preexisting mRNA (Darnell et al., 1971). The need for several adenylic acid residues at the 3′-end of mRNA for its proper processing has provided an important functional role for this enzyme. Such a role for the mitochondrial poly(A) polymerase is now imminent following several reports on the presence of a poly(A) sequence at the 3′ terminus of mitochondrial mRNA (Perlman et al., 1973; Ojala and Attardi, 1974a,b). Moreover, a mitochondrial synthesis of functional mRNA has now been well documented in HeLa (Perlman et al., 1973) and in yeast (Mahler and Dawidowicz, 1973). It is therefore conceivable that mitochondrial poly(A) polymerase adds on adenylic acid units to preexisting mitochondrial mRNA. The preference of mitochondrial RNA over nuclear RNA as a primer by the mitochondrial poly(A) polymerase strongly suggests that in vivo this enzyme adds poly(A) to mitochondrial mRNA.

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