Purification and Properties of Mitochondrial Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from Ovaries of *Xenopus laevis*†

Guang-Jer Wu and Igor B. Dawid*

ABSTRACT: DNA-dependent RNA polymerase was solubilized from ovarian mitochondria of *Xenopus laevis* and purified about 500-fold to a specific activity of over 8000 pmoles of UTP incorporated per mg of protein in 20 min. The enzyme requires magnesium ions, is neither stimulated nor inhibited by manganese ions, is strongly inhibited by monovalent salt, and is stimulated by spermine or spermidine and by dithiothreitol. In the presence of KCl at concentrations of 0.1 M or above the mtRNA polymerase sediments at 6.2 S. In low salt the enzyme sediments in the form of oligomers with a predominant peak at 60 S. Gel electrophoresis in the presence of sodium dodecyl sulfate reveals a predominant polypeptide with a molecular weight of 46,000. We suggest that the mitochondrial polymerase contains a single type of polypeptide chain; the "high-salt form" of the enzyme (6.2 S form) may be a dimer or trimer of this polypeptide. The polymerase is insensitive to α-amanitin and to three derivatives of rifamycin up to a concentration of 100 μg/ml. The enzyme is inhibited by three other rifamycin derivatives by 95% at 50 μg/ml. The mitochondrial polymerase prefers denatured over native nuclear DNA (nDNA) as template, but prefers native closed-circular mtDNA over nicked or denatured forms of this DNA. The best template tried is synthetic poly[d(A-T)]. By a variety of criteria the mtRNA polymerase is distinct from *Escherichia coli* RNA polymerase and from the three known nRNA polymerases of *X. laevis*.

The presence of organelle-specific DNA and RNA in mitochondria (see Borst, 1972) suggested that RNA polymerase might also be found in these particles. Whole mitochondria had been known to incorporate nucleoside triphosphates into RNA (Wintersberger, 1964). The solubilization of mtRNA[1] polymerase, its purification, and its characterization were achieved with the enzyme from *Neurospara* (Küntzel and Schäfer, 1971) and yeast (Tsai *et al.*, 1971). RNA polymerizing activity has also been solubilized from rat liver mitochondria (Reid and Parsons, 1971; Gadaleta *et al.*, 1970).

Mitochondria from the ovaries of *Xenopus laevis*, the South African clawed toad, have been a useful source of mtDNA (Dawid and Wolstenholme, 1967), RNA (Dawid, 1972; Dawid and Chase, 1972), and ribosomes (Swanson and Dawid, 1970). In this paper we report the isolation and characterization of mtRNA polymerase from ovaries of *X. laevis*. The properties of the isolated mitochondrial enzyme distinguish it clearly from the three known nRNA polymerases of *X. laevis* (Roeder *et al.*, 1970) and from bacterial polymerase (Burgess, 1969).

Materials

The source of materials was as follows: α-amanitin, the bicyclic polypeptide toxin from *Amanita phalloides*, C. F. Boehringer; ribonuclease-free pancreatic DNase I and pancreatic RNase, Worthington; ammonium sulfate (special enzyme grade), Mann; tritiated UTP ([5-^{3}H]UTP, 13 Ci/mmole) Schwarz and Mann BioResearch; phosphocellulose (P-11), DEAE-cellulose (DE-52, microgranular), and CM-cellulose (CM-52, microgranular), Reeve-Angel; DEAE-Sephadex (A-25), Pharmacia; rifamycin derivatives, Calbiochem, and Dr. Silvestri, Lepetit.

E. coli DNA-dependent RNA polymerase, prepared by the method of Burgess (1969) was a gift of D. D. Brown. mtDNA was isolated from *X. laevis* ovaries (Dawid, 1966; Dawid and Wolstenholme, 1967). Component I (closed-circular, or twisted-circular mtDNA) was purified by sucrose gradient sedimentation as the 39S component. The DNA was further purified by centrifugation in CsCl gradients and dialyzed.

Methods

Buffers. The following buffers were used: TGED, 0.05 M Tris·HCl (pH 7.9)–0.1 mM sodium EDTA–25% (v/v) glycerol–0.5 mM dithiothreitol; TED, TGED buffer without glycerol; STE, 0.25 M sucrose–0.03 M *N*-tris(hydroxymethyl-methyl-2-aminoethanesulfonic acid–0.5 mM NaEDTA (pH 8.0); ST, STE without EDTA; KMTD, 0.125 M KCl–0.007 M $MgCl_2$–0.03 M Tris·HCl (pH 8.0)–0.03 mM dithiothreitol; MSH, 0.22 M D-mannitol–0.07 M sucrose–0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, sodium salt (pH 7.5).

RNA Polymerase Assay. The assay mixture (40 μl) contains 0.05 M Tris·HCl (pH 8.0), 0.01 M $MgCl_2$, 2.5 mM dithiothreitol, 0.5 mg of bovine serum albumin/ml, 0.5 mM spermine, 0.5 mM each of ATP, GTP, and CTP, 4 μCi (0.3 nmole/40 μl or 7.5 μM) of [^{3}H]UTP, 5 μg of heat-denatured calf thymus DNA (unless specified), and enzyme. These conditions were also optimal for the incorporation of UMP into RNA by whole mitochondria, except that no DNA was needed. Higher concentrations of UTP at lower specific activities were used in somes cases and will be specified in the text. All assays were carried out at 30° for 20 min unless otherwise specified. Reactions were terminated by pipetting aliquots of the assay

† From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210. *Received May 25, 1972.* A preliminary account of parts of this work has appeared (Wu and Dawid, 1972).

[1] Abbreviations used are: mtDNA, mitochondrial DNA; DEAE-cellulose, diethylaminoethylcellulose; CM-cellulose, carboxymethylcellulose; nDNA, nuclear DNA; PC enzyme, phosphocellulose-purified enzyme.

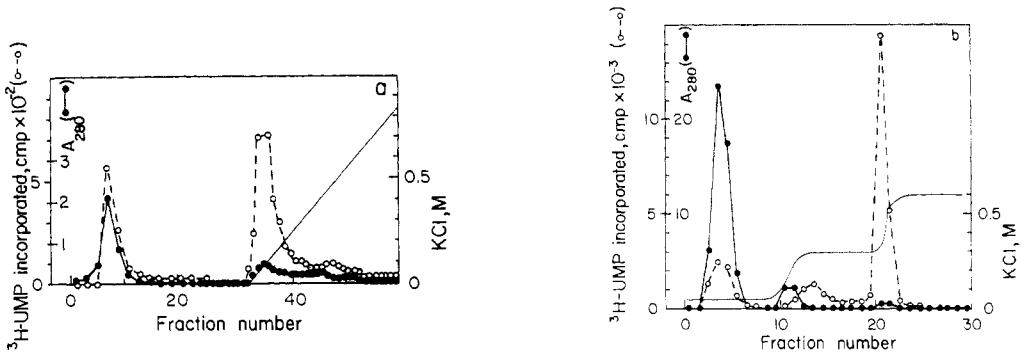

FIGURE 1: Column chromatography of mtRNA polymerase. (a) DEAE-Sephadex profile. Ammonium sulfate precipitate (8 mg) was applied to a column (0.6 × 5.0 cm) of A-25 at 3 ml/hr. The column was washed with TGED at a flow rate of 12 ml/hr. Fractions of 0.5 ml each were collected for the first 12 fractions, and fractions of 0.25 ml after this. Two microliters from each fraction were used for assay. (b) Preparative phosphocellulose column. Protein (240 mg) was applied to a column (1.8 × 20 cm) and eluted as described in Methods. Fractions of 6.5 ml were collected and 2 μl from each were assayed.

mixture onto Whatman No. 1 paper. The filters were washed in trichloroacetic acid at 0–4° to remove unincorporated [^{3}H]UTP (Bollum, 1968). The dried filters were counted in 5 ml of toluene-based fluor. Blank values were obtained from reactions without enzyme.

Isolation of Mitochondria from Ovaries of X. laevis (modified from Dawid, 1966, and Swanson and Dawid, 1970). All operations were performed at 0–5°. Buffers were autoclaved or sterilized by filtration. Whole ovaries of *X. laevis* were homogenized in ten volumes of STE in a motor-driven loose Teflon-glass homogenizer at low speed. The homogenate was filtered through cheesecloth. The yolk platelets and most of the pigment granules were pelleted at 1500 rpm for 15 min in an International 259 centrifuge head. Mitochondria and some pigment granules were pelleted at 10,000 rpm for 20 min in a Sorvall centrifuge, and were washed once in STE, twice with KMTD, once again with STE buffer, and finally with ST buffer. The mitochondria were suspended in ST buffer at 20 mg/ml.

Purification of mtRNA Polymerase. All operations were carried out at or close to 0°. Mitochondria (640 mg of protein) suspended in ST were pelleted and resuspended in MSH buffer containing 2% (w/v) digitonin to a protein concentration of 10–15 mg/ml. The suspension was homogenized in a Dounce glass homogenizer with 50 strokes and incubated for 20 min at 0°. The solution was centrifuged in a Spinco No. 65 fixed-angle rotor at 60,000 rpm for 30 min, and the clear amber supernatant was collected.

Ammonium sulfate was added slowly with stirring to 62.5% of saturation. The pH was kept above 7.0 by adding 4 M NH_4OH. After adding the ammonium sulfate, the solution was stirred for 30 min at 0°. The precipitate was collected by centrifugation at 60,000 rpm for 60 min. The pellet was dissolved in TGED and dialyzed against 100 volumes of TGED with two changes overnight.

KCl was added to the dialyzed solution to give the same conductivity as that of TGED containing 0.05 M KCl. The solution was applied to a phosphocellulose column (1.8 × 20 cm) at a flow rate of 30 ml/hr. The column was washed with one column volume of TGED (containing 0.05 M KCl) and three column volumes of TGED (containing 0.3 M KCl) and the enzyme was eluted with three column volumes of TGED (containing 0.6 M KCl). Fractions, 6.5 ml each, were collected and small aliquots were assayed for activity. Fractions containing RNA polymerase activity were pooled and dialyzed against 100 volumes of TGED. The dialyzed enzyme solution was divided into small portions and stored at −70°. Enzyme at this stage (PC enzyme) was used in many of the experiments to be reported.

Glycerol gradient centrifugation was used as a final purification step in some cases. PC enzyme was diluted with two volumes of TED, and made 0.2 M KCl and 8% in glycerol. This solution (1 ml) was layered on each of six 11-ml 10–30% glycerol gradients containing TED buffer and 0.2 M KCl. The gradients were centrifuged at 41,000 rpm in a Beckman SW-41 rotor for 5 hr. Fractions containing enzymatic activity were pooled and dialyzed against TGED. To concentrate the enzyme the solution was layered over 0.3 ml of glycerol and centrifuged in a SW-65L rotor at 60,000 rpm for 15 hr. Fractions of 0.25 ml were collected from the bottom and small aliquots were assayed for activity.

Analytical Glycerol Gradient Sedimentation. Samples of 0.35 ml containing 8% glycerol were layered onto 4.5-ml linear 10–30% gradients and centrifuged in a Beckman SW-65L rotor at −2°. For the determination of sedimentation coefficients of RNA polymerase which had been disaggregated with KCl, the following proteins were used as standards: *E. coli* RNA polymerase (13.0 S, 450,000; Richardson, 1969; King and Nicholson, 1971), yeast alcohol dehydrogenase (7.6 S, 150,000; Martin and Ames, 1961), and bovine hemoglobin (4.3 S, 64,600; Braunitzer *et al.*, 1964).

Polyacrylamide Gel Electrophoresis. Subunit structure of the RNA polmerase was studied by electrophoresis in 10% polyacrylamide gels in the presence of the dodecyl sulfate (Wu and Bruening, 1971). Bovine serum albumin (mol wt 66,000), ovalbumin (mol wt 47,000), and horse heart cytochrome *c* (mol wt 13,400) were used as standards.

Results

Comments on the Procedure for the Isolation of mtRNA Polymerase. The method of preparation of mitochondria affects their ability to incorporate nucleoside triphosphates into RNA and the facility of extracting polymerase. Either STE or KMTD alone was less favorable for the isolation of mitochondria than the procedure described here in which both buffers were used.

Several methods were tried for the solubilization of RNA polymerase from mitochondria. Homogenization in 0.5 M KCl and treatment with DNase (Tsai *et al.*, 1971), grinding

TABLE I: Purification of mtRNA Polymerase from *X. laevis* Ovaries.

Steps	Protein (mg)	Total Act. (Units)[a]	Sp Act. (Units/mg)	Yield (%)
1. Whole mitochondria	640	1.0×10^4	16	100
2. Digitonin-treated mitochondria	640	5.2×10^4	80	520
3. High-speed supernatant	320	4.5×10^4	150	450
4. Ammonium sulfate precipitate[b]	250	8.6×10^4	350	860
5. Phosphocellulose column	8	3.2×10^4	3900	320
6. Glycerol gradient	1	0.8×10^4	8630	82

[a] One unit of activity of enzyme incorporates one $\mu\mu$mole of UTP in 20 min under the conditions described in Methods except that the UTP concentration was 4.3 nmoles/40 μl or 0.1 mM. All assays were done under conditions where activity was a linear function of the amount of protein present (see also Figure 2). Protein was determined by the biuret method (Gornall *et al.*, 1949), or by the absorbance at 280 and 260 nm (Warburg and Christian, 1941). [b] After dialysis.

with alumina (Meyer and Simpson, 1970), freezing and thawing in hypotonic medium (Kalf and Ch'ih, 1968), sonication in high-salt buffer (Roeder and Rutter, 1969) or at low salt (Küntzel and Schäfer, 1971), and treatment with various detergents (Triton X-100, sodium cholate, sodium deoxycholate, Brij-58, Lubrol, and NP-40) were explored. All these methods were abandoned, either due to poor yield (10–40% relative to whole mitochondria) of the RNA polymerase activity in the soluble fraction or inactivation of the enzyme.

Digitonin proved the most effective means of solubilization of RNA polymerase. Concentrations of digitonin 2% or above solubilize more than 80% of the activity. The total apparent activity in the soluble fraction was fivefold higher than in whole mitochondria. This may be due to removal of a permeability barrier against either nucleoside triphosphates or added DNA (the synthetic activity of whole mitochondria is not affected by added DNA).

Several chromatographic procedures were explored. mt-RNA polymerase purified through the ammonium sulfate step is not absorbed to CM-cellulose. It absorbed to DEAE-cellulose or DEAE-Sephadex (A-25) if applied in TGED buffer without KCl. However, the bulk of the enzymatic activity eluted at 0.1 M KCl, giving little purification. Figure 1a shows an example using A-25 DEAE-Sephadex. Phosphocellulose proved a useful chromatographic material using a salt gradient (not shown) or with step elution (Figure 1b). The glycerol gradient step in polymerase purification is based on the salt-dependent reversible disaggregation of the enzyme (see below).

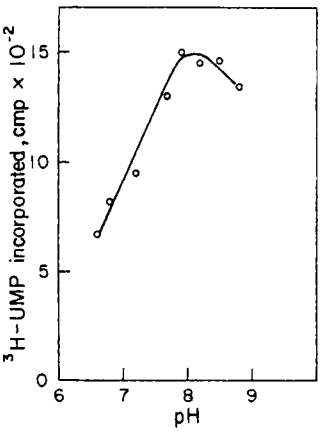

FIGURE 3: Effect of pH on enzyme activity. Tris-HCl buffers were used at different pH in the standard assay.

Table I summarizes the yield and purification obtained at each step. The increase in activity at step I has been mentioned above. The increase upon ammonium sulfate precipitation may be due to removal of nucleases, but this point has not been studied in detail. A major loss in yield occurs at the glycerol gradient step. This loss is likely to be caused by the fact that the gradient step takes a period of time corresponding to 1–2 half-lives of the enzyme at 0–4° (see below).

Requirements and Some Properties of mtRNA Polymerase. All enzyme assays were carried out in a range where activity depended linearly on protein concentration. An example is shown in Figure 2 for PC enzyme. The kinetics of the reaction depend on the template and will be presented below.

The effect of the pH on activity is shown in Figure 3; a broad optimum was found around pH 8.0. The enzyme was dependent absolutely on magnesium ions (Figure 4). The

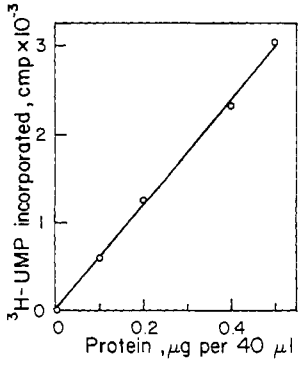

FIGURE 2: Protein dependence of the activity of PC enzyme. The standard assay was incubated for 20 min.

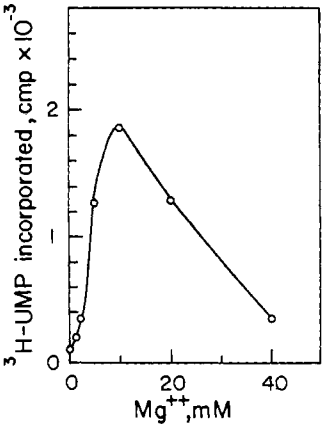

FIGURE 4: Requirement for magnesium ions of the enzyme activity.

TABLE II: Requirements of the mtRNA Polymerase.

Omission or Addition	Act. (%)
Complete[a]	100
−Spermine	36
−Bovine serum albumin	57
−ATP, GTP, CTP	2
−mtDNA	3
−Dithiothreitol	70
+DNase I (125 μg/ml)	2

[a] The complete system is described in Methods but contained native mtDNA (0.75 μg). The control reaction incorporated 555 cpm. The enzyme was purified through the phosphocellulose step.

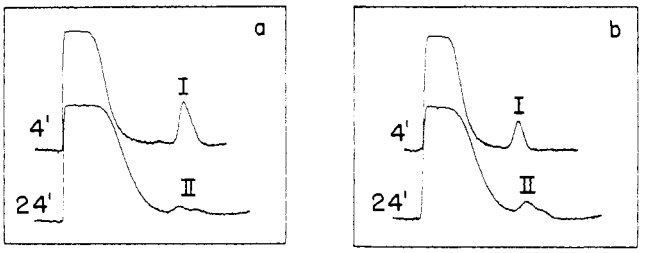

FIGURE 6: Endonuclease assay in mtRNA polymerase. A standard assay reaction (40 μl) was assembled with 2.3 μg of mtDNA, component I, and 2 μg of PC enzyme, At zero time 10 μl was mixed with 1 μl of 1 N KOH and analyzed by band sedimentation over 2.5 M CsCl–0.05 N KOH (a); the proportion of component I is 80%. After 1-hr incubation at 30° another 10 μl was analyzed in the same (b); component I amounts to 55%. In each case one exposure is shown 4 min after reaching 40,000 rpm, and a second exposure after 24 min. The short time displays component I_{Alk} sedimenting at 87 S, the later exposure shows component II_{Alk} which includes single-strand linear and circular molecules and sediments at 22 S.

activity was not significantly affected by manganese ions added to reactions containing magnesium, but manganese does not replace magnesium. Sulfhydryl reducing agents, like dithiothreitol, and polyamines, like spermine and spermidine, stimulate the activity (Table II). The enzyme is dependent on added DNA and on nucleoside triphosphates, and was severely inhibited by DNase (Table II). Bovine serum albumin was added routinely to the assay mixture to guard against denaturation of the enzyme.

The dependence of activity on the UTP concentration was measured at pH 8.0. The K_m for UTP was 0.02, or 0.015 mM, with heat-denatured calf thymus DNA and native mtDNA as template, respectively.

mtRNA polymerase is strongly inhibited by salt. Figure 5 shows that the addition of salt to as little as 20 mM leads to a significant inhibition of the enzyme. This inhibition is reversible upon removal of the salt.

The purified polymerase has moderate stability. Its half-life at 0–4° is about 24 hr. At 30° the half-life is about 1–3 hr, and at 41° inactivation is complete in 10 min. The enzyme has been stored in TGED for at least 1 year at −70° without loss of activity.

Enzyme preparations at different stages of purity were tested for the presence of nucleases. For this purpose the enzyme was incubated under assay conditions but without [^{3}H]UTP with radioactive DNA or RNA for periods up to 22 hr, and the release of acid-soluble material was measured. Although the ammonium sulfate precipitate contained much DNase and RNase activity, the PC enzyme contained no measurable RNase and only a trace of DNase activity. The glycerol gradient step removed the last trace of DNase from the RNA polymerase. A particularly sensitive assay for endonuclease activity is the conversion of closed-circular mtDNA (component I) to the open form (component II), since this conversion requires the breakage (nicking) of only one phosphodiester bond in about 34,000. Figure 6 shows that a 1-hr incubation at 30° with 2 μg of PC enzyme lead to a small reduction in the proportion of component I, but more than half of the mtDNA had remained in the closed form. A similar result was obtained with glycerol gradient enzyme. These experiments are the basis for a meaningful use of component I as a template for the polymerase (see below) since component I does survive for some time in the reaction and can thus act as template.

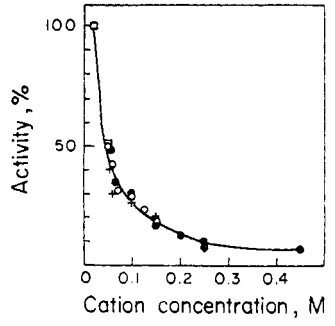

FIGURE 5: Inhibition of mtRNA polymerase by salt. The 100% point represents a reaction which contained 5 mM Tris buffer instead of the usual 50 mM. All other reactions were standard assays (see Methods). Squares refers to reactions containing Tris as the only monovalent cation. To the other reactions, KCl (open circles), NaCl (closed circles), or ammonium sulfate (triangles) was added to a total cation concentration given by the abscissa.

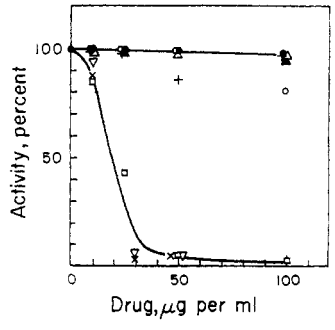

FIGURE 7: Effect of drugs on RNA polymerase activity. Each reaction contained 1 μg of PC enzyme, 1.3 μg of mtDNA component II, and different concentrations of drugs. Points were taken at 20, 40, and 60 min; the kinetics of all reactions were similar and the values at the three time points were averaged. Activity is expressed as per cent of activity in the absence of drugs. Rifampicin (●), rifampin (△), rifamycin SV (+), 3-(4-benzyl-2,6-dimethylpiperazinoiminomethyl)rifamycin SV (AF/ABDP) (□), α-amanitin (○), 3-formylrifamycin SV *O*-(diphenylmethyl)oxime (AF/05) (▽), and 3-formylrifamycin SV *O*-*n*-octyloxime (AF/013) (×).

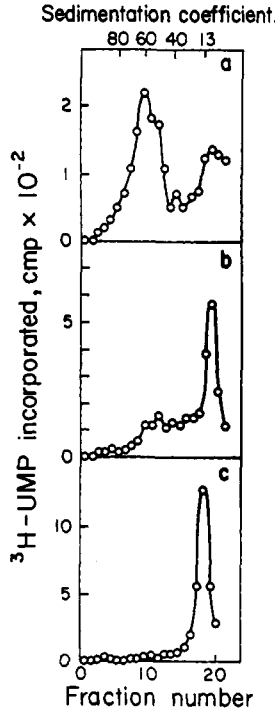

FIGURE 8: Aggregation of mtRNA polymerase. The enzyme was analyzed in glycerol gradients in TED buffer with (a) no addition, (b) 0.05 M KCl, and (c) 0.1 M KCl. The sedimentation coefficients indicated on the abscissa were estimated from parallel gradients containing *X. laevis* ribosomes and ribosomal subunits (80, 60, and 40 S), and *E. coli* RNA polymerase. Sedimentation was carried out in the Beckman SW65 rotor for 1.5 hr at 60,000 rpm and −2°.

Sensitivity to Drugs. Rifamycin and some of its derivatives were tested for their effect on the activity of mtRNA polymerase. Figure 7 shows that rifampin, rifamycin SV, and rifampicin do not inhibit the enzyme up to a concentration of 100 μg/ml. *E. coli* RNA polymerase was inhibited by these compounds by at least 95% at 1 μg/ml. Three complex rifamycin derivatives [3-(4-benzyl-2,6-dimethylpiperazinoiminomethyl)rifamycin SV[2] (Lepetit code AF/ABDP), 3-formylrifamycin SV *O*-(diphenylmethyl)oxime (AF/05), and 3-formylrifamycin SV *O*-*n*-octyloxime (AF/013)] did inhibit the polymerase at moderately high concentrations (Figure 7).

α-Amanitin, a potent inhibitor of the nRNA polymerase II from different organisms including *X. laevis* (Roeder *et al.*, 1970), has no effect on the mitochondrial enzyme (Figure 7).

Sedimentation Properties of the Polymerase. Sedimentation in glycerol gradients revealed that the enzyme aggregates at low ionic strength (Figure 8). In the salt concentrations used in the standard assay the enzyme sediments in multiple forms, with a major peak at 60 S (Figure 8a). Increasing concentrations of KCl lead to dissociation (Figure 8b,c). No further change was seen in KCl concentrations above 0.1 M.

The sedimentation coefficient of the high-salt form was measured by comparison to known proteins (see Methods) in gradients run for a longer time. The result was a value of 6.2 S for the "high-salt monomer" of the mitochondrial enzyme. This value alone does not allow the calculation of a molecular weight, but a value of 100,000–150,000 may be a probable estimate.

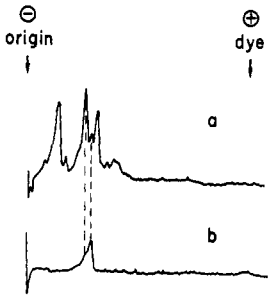

FIGURE 9: Gel electrophoresis of mtRNA polymerase in the presence the dodecyl sulfate. Tracings of stained gels of 60 μg of PC enzyme (a) and 30 μg of glycerol gradient enzyme (b) are shown.

Gel Electrophoresis of mtRNA Polymerase. Because of aggregation of the enzyme at low ionic strength electrophoresis had to be carried out in the presence of high salt or sodium dodecyl sulfate. PC enzyme showed several bands in the dodecyl sulfate gels (Figure 9a) while glycerol gradient enzyme formed a single band with a shoulder (Figure 9b). It is unlikely that any of the polypeptides removed from the preparation by the glycerol gradient step is a true component of the enzyme since the specific activity increased during this step (Table I). Since the shoulder in the glycerol gradient enzyme corresponds to a major peak in the PC pattern which was much reduced in the last purification step, we believe it more likely to be a contaminating protein than a subunit of the polymerase. If this interpretation is correct mtRNA polymerase may contain a single type of subunit (see Discussion). The molecular weight of this polypeptide is 46,000, as determined by comparison to the electrophoretic mobilities of polypeptides of known size in the dodecyl sulfate gels (see Methods). The molecular weight of the minor component (shoulder) is 50,000.

Template Specificity of mtRNA Polymerase. The effectiveness of different templates during a 20-min incubation is shown in Figure 10. Great differences were observed. mtDNA was most effective in the native closed-circular form, *i.e.*, component I. Component II, *i.e.*, mtDNA containing an average of one nick per molecule, has reduced efficiency. mtDNA with an average of five nicks per molecule ("old component II") had strongly decreased template efficiency in the native form. However, denatured mtDNA (symbolized in the figure by Δ) was more efficient in the multiply nicked form. The right half of Figure 10a shows that nDNA from calf thymus was an efficient template in the denatured form only. The same is true for nDNA from *X. laevis* (not shown). *E. coli* DNA was a poor template in either form. Poly[d(A-T)] was the most effective template tried (Figure 10b). This observation agrees with findings on *Neurospora* mtRNA polymerase (Küntzel and Schäfer, 1971).

Figure 11 shows the kinetics of the reaction with different forms of mtDNA as template. The high template efficiency of component I is even more apparent after longer reaction times.

[2] The compound 3-(4-benzyl-2,6-dimethylpiperazinoiminomethyl)-rifamycin SV (AF/ABDP) has also been described as 2,6-dimethyl-4-*N*-benzyldemethylrifampicin (AF/ABDMP); it has also been described as 2,5-dimethyl-4-*N*-benzyldemethylrifampicin (Gurgo *et al.*, 1971). According to a personal communication from Dr. Silvestri of Gruppo Lepetit the 2,6-dimethyl formula is the correct one, and the compounds described above are all identical.

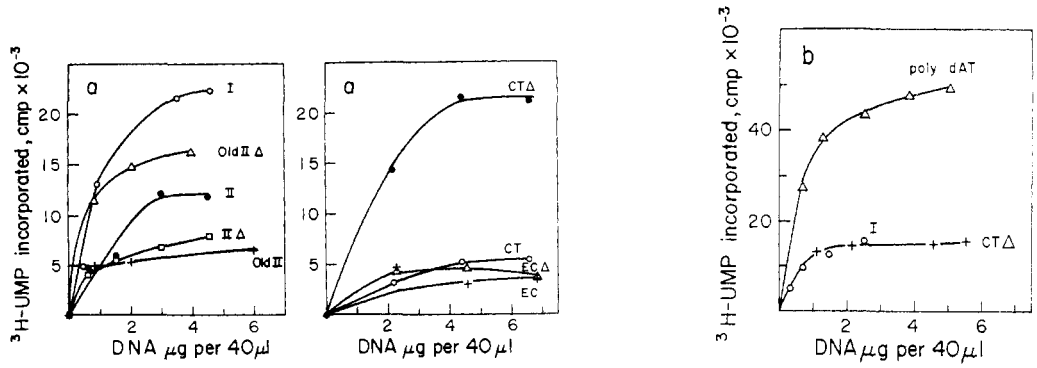

FIGURE 10: Template saturation of mtRNA polymerase. PC enzyme (1 μg) was tested in the standard assay for 20 min. The different DNA preparations were: I, *X. laevis* mtDNA component I; II, mtDNA component II (with an average of one nick per molecule); old II, preparation of component II after storage for several months with an average of five nicks per molecule; CT, calf thymus (nuclear) DNA; EC, *E. coli* DNA; Δ refers to DNA preparations denatured by heating. The experiments of part a of this figure were done with one sample of enzyme. In part b a separate preparation of enzyme was used to compare the template efficiency of mtDNA component I (○), denatured calf thymus DNA (+), and poly[d(A-T)] (△).

Discussion

The enzyme isolated from *X. laevis* mitochondria is a typical DNA-dependent RNA polymerase, requiring template, four nucleoside triphosphates, and magnesium ions. The enzyme appears to contain a single type of polypeptide chain of a molecular weight of 46,000. The active form is apparently an oligomer which dissociates in solutions containing 0.1 M KCl or higher to a form sedimenting at 6.2 S. This high-salt form could consist of a single chain of 46,000, but more likely might be a dimer or trimer. An alternate explanation must be considered, however. The specific activities of the mitochondrial polymerases from *X. laevis*, *Neurospora* (Küntzel and Schäfer, 1971), and yeast (Tsai *et al.*, 1971) are all similar to each other and at least 20-fold lower than the specific activity of *E. coli* polymerase (Burgess, 1969). This fact allows two interpretations: either mitochondrial polymerases are inherently less active, or the current preparations are still quite impure. In the latter case the main polypeptide of our preparation, which has a molecular weight of 46,000 may not be a component of the enzyme but a contaminant. This situation does not appear likely but cannot be excluded at present.

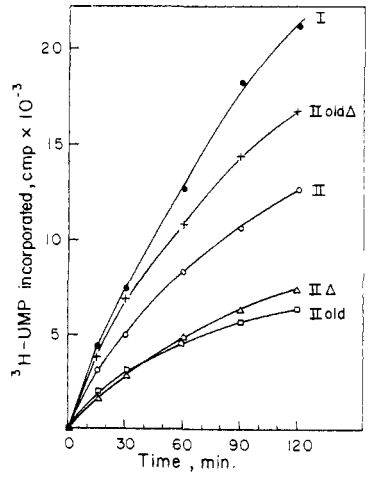

FIGURE 11: Kinetics of the mtRNA polymerase reaction. Each DNA was at a concentration of 2.1 μg/40 μl. The other conditions and meaning of symbols are as in Figure 10.

There are no suggestions for factors or subunits that might have been removed from the enzyme during purification. When ammonium sulfate precipitated enzyme which had not been subjected to phosphocellulose chromatography was analyzed on glycerol gradients, its sedimentation behavior was the same as that reported for the PC enzyme. The addition of fractions of the large peak of proteins emerging early from a phosphocellulose column to the purified polymerase did not lead to a stimulation of the activity.

X. laevis mitochondrial polymerase is distinct in several ways from bacterial and from nuclear enzymes. It differs from *E. coli* RNA polymerase (Burgess, 1969) in size, subunity structure, behavior in chromatography, and inhibition by salt. Three polymerases have been described in the nuclei of various eucaryotes (Roeder and Rutter, 1969), including *X. laevis* (Roeder *et al.*, 1970; Roeder, 1972; R. G. Roeder, personal communication). Mitochondrial polymerase is different from all three nuclear polymerases in its behavior on columns of DEAE-Sephadex, CM-cellulose, and phosphocellulose. Furthermore, nuclear polymerases II and III are activated by salt, and polymerase I is not influenced by salt up to a cation concentration of about 0.06 M, while mitochondrial polymerase is strongly inhibited (Figure 5). All three nuclear polymerases are more active with manganese ions than with magnesium, but this is not the case with the mitochondrial enzyme. Nuclear polymerase II is inhibited by α-amanitin while mitochondrial polymerase is not affected. These differences clearly show that the enzyme isolated from frog ovarian mitochondria is distinct, and eliminate the possibility that the enzyme obtained is actually one of the nuclear polymerases which contaminated the mitochondrial preparations.

mtRNA polymerases have been solubilized and purified to varying degrees from several other organisms. The properties of the various mitochondrial enzymes appear rather diverse. The enzyme from *Neurospora* has been purified to homogeneity; it aggregates in low salt, sediments at 6.3 S in high salt, and contains a single polypeptide chain of a molecular weight of 64,000 (Küntzel and Schäfer, 1971). These physical properties are rather similar to those of the *X. laevis* enzyme. At least 2 mtRNA polymerases have been isolated from yeast (Tsai *et al.*, 1971). These enzymes are different from the frog polymerase in their behavior on DEAE-Sephadex columns. Insufficient data are available for a comparison

of physical properties. RNA polymerase from rat liver mitochondria has been solubilized (Gadaleta *et al.*, 1970; Reid and Parsons, 1971).

The question of rifamycin sensitivity of mtRNA polymerases has attracted considerable attention, especially in view of the frequently stressed similarity of mitochondria and procaryotes. *Neurospora* polymerase is rifampicin sensitive (Küntzel and Schäfer, 1971), the yeast enzyme is resistant (Tsai *et al.*, 1971), and rat liver polymerase may be sensitive (Gadaleta *et al.*, 1970; Reid and Parsons, 1971). Some of these discrepancies may be explained by our results which show that rifamycin sensitivity of mitochondrial polymerase is a relative rather than absolute property of the enzyme and depends on the type of derivative and the concentration used. Three derivatives of rifamycin were inactive up to a concentration of 100 μg/ml, while three complex derivatives did inhibit *X. laevis* mitochondrial polymerase (Figure 7). The derivative AF/ABDP inhibited *E. coli* RNA polymerase to 50% at a concentration of 0.1 μg/ml, *i.e.*, the mitochondrial enzyme is about 100-fold less sensitive than the bacterial enzyme. The same derivative (AF/ABDP)[2] is an inhibitor of RNA-dependent DNA polymerase from tumor viruses, with 50% inhibition achieved at about 50 μg/ml (Gurgo *et al.*, 1971). When several rifamycin derivatives at different concentrations are tested with mitochondrial polymerases from different sources, a more consistent pattern may emerge. But it seems already clear that the enzyme from *Neurospora* is more sensitive than the enzyme from *X. laevis*.

X. laevis mtRNA polymerase uses different templates with highly variable efficiency. Poly[d(A-T)] is a highly efficient template, as it is for mitochondrial polymerase of *Neurospora* (Küntzel and Schäfer, 1971). Among natural native DNAs intact frog mtDNA (component I) is the best template. However, denatured calf thymus DNA is about equally effective. With mtDNA as template the enzyme shows strand selectivity: the heavy strand is transcribed preferentially from native mtDNA, and the light strand from denatured mtDNA (G. J. Wu and I. B. Dawid, unpublished data). These facts suggest that specific recognition by the mtRNA polymerase of its natural template might be achieved *in vitro*.

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