Purification of components required for accurate transcription of ribosomal RNA from *Acanthamoeba castellanii*

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ABSTRACT

The components required for specific transcription of ribosomal RNA were isolated from logarithmically growing *Acanthamoeba castellanii*. The transcription initiation factor fraction, TIF, and RNA polymerase I were extracted from whole cells at 0.35 M KCl. The extract was fractionated with polyethylenimine, then chromatographed on phosphocellulose (P11) which resulted in the separation of TIF from RNA polymerase I. The fractions containing TIF were further chromatographed on DEAE cellulose (DE52), Heparin Affigel, and Matrex green agarose, followed by sedimentation through glycerol gradients. TIF was purified approximately 17,000-fold, and shown to have a native molecular weight of 289 kD, and to bind specifically to rRNA promoter sequences by DNase I footprinting. The addition of homogeneous RNA polymerase I to this complex permitted the initiation of specific transcription *in vitro*. The phosphocellulose fractions containing RNA polymerase I were chromatographed on DEAE cellulose, Heparin-Sepharose, DEAE-Sephadex, and sedimented through sucrose gradients. Polymerase I was purified to apparent homogeneity with a yield of 8.1% and a specific activity of 315. It contained one fewer subunit than previously reported. DNase I protection experiments demonstrated that in both partially purified and homogenous fractions, RNA polymerase I was capable of stable binding to the TIF-rDNA complex, and correctly initiating transcription on rDNA templates.

INTRODUCTION

In order to determine the molecular mechanism of eukaryotic transcription, it is necessary to identify and purify the cellular components involved. The large precursor ribosomal RNA (rRNA) genes are transcribed by RNA polymerase I. This enzyme has been purified from a variety of sources, and is unable to accurately transcribe purified ribosomal DNA (rDNA) templates without additional cellular components [reviewed in (1–5)]. To analyze the mechanism of rRNA synthesis, *in vivo*

and cell-free transcription systems have been developed [reviewed in (6,7)]. These systems have been used to delineate both promoter sequences and *trans*-acting factors needed to direct accurate transcription by RNA polymerase I.

A common mechanism involved in rDNA transcription is the stable binding of *trans*-acting factors to rDNA templates prior to initiation (8,9) upstream of the transcription start site (10). Though highly conserved sequences generally are not observed in rRNA genes from different organisms, rDNA promoters are organized in a similar manner [reviewed in (6)]. Sequences proximal to the transcription start site, denoted as the core promoter, extend from approximately -40 to +5. The core promoter is essential for accurate initiation of transcription, and has been shown to be involved in the formation of stable preinitiation complexes. Further upstream, between -110 and -150 are sequences that augment transcription, apparently by influencing stable binding of *trans*-acting factors or RNA polymerase I to the core promoter (11–14). In several species, regions in the non-transcribed spacers of rRNA genes up to 2 kilobases upstream of the transcription start site have been found to function as enhancers (15–21).

Trans-acting factors that bind to these regions of the rDNA promoter have been purified from several sources. Factors that bind to the core promoter have been identified (10,22–26) and cloned (27–30). These factors have been shown to be essential for the precise initiation of transcription. Other components have been identified that bind to the intergenic spacer regions of the rDNA repeats and stimulate transcription in a manner similar to enhancer proteins [(21,26) reviewed in (31)].

Studies performed in the lower eukaryotic organism *Acanthamoeba castellanii* have demonstrated that at least one *trans*-acting factor, TIF, is involved in both stable complex formation and accurate initiation of transcription *in vitro* (10,32). The *Acanthamoeba* promoter consists of DNA sequences extending from -47 to +8 (33). While sequences from -31 to +8 are sufficient for transcription *in vitro*, sequences between -47 and -17 have been shown to be essential for the formation of a stable complex with TIF (10). DNase I footprinting studies have demonstrated that TIF binding protects a larger region, extending from -69 to -12 (32,34).

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In this study, the procedure is described used to isolate from *Acanthamoeba* whole cell extracts both the TIF and the RNA polymerase I used in the above studies. TIF and the three classes of nuclear RNA polymerase were isolated from the same initial whole cell extract. By additional chromatographic and sedimentation steps, TIF was purified approximately 17,000 fold, and RNA polymerase I was purified to homogeneity. TIF was shown to stably interact with the rDNA promoter and direct accurate initiation of transcription when complemented with RNA polymerase I.

MATERIALS AND METHODS

Reagents

Unlabelled nucleoside triphosphates were from P-L Biochemicals; [γ - 32 P] ATP (3000 Ci/mmol) was from New England Nuclear; [5,6- 3 H]-UTP (35 Ci/mmol) was from ICN Biomedicals; DE81 filter discs were from Whatman; Coomassie Brilliant Blue (protein assay dye) was from Bio-Rad Laboratories; polyethyleneimine was from Eastman Kodak; α -amanitin was from Boehringer Mannheim Biochemicals. Phosphocellulose (P11) and DEAE-cellulose (DE-52) were from Whatman; Heparin-Sepharose, DEAE-Sephadex, and Sepharose CL-4B were from Pharmacia, Inc.; Heparin agarose and Biorex 70 were from Bio-Rad Laboratories; and Matrex green agarose was from Amicon. Resins were pre-cycled according to manufacturers' recommendations, equilibrated in the appropriate buffer, and stored at 4°C prior to use.

Cell growth

Acanthamoeba castellanii cells were grown to a density of 5×10^6 cells/ml (midlog) and harvested as described previously (35). Cells were washed in a buffered solution containing 10 mM Tris-HCl (pH 7.9) and 0.15 M KCl, centrifuged at $10,000 \times g$ for 10 min and quick-frozen by dripping cells into liquid nitrogen.

DNA templates

The plasmid pSBX60 was used as a template for the *in vitro* transcription assays. This plasmid contains the *Acanthamoeba* ribosomal RNA promoter and initiation site cloned into the pBR322 *Xma* III site (33). The plasmid was linearized with *Ava* I, and produced a runoff RNA transcript of 500 nucleotides.

RNA polymerase assays

Non-specific transcription assays were performed as described previously (35). RNA polymerase activity was assayed in the presence of either 1 μ g/ml of α -amanitin, which inhibits RNA polymerase II, or 600 μ g/ml of α -amanitin which inhibits both RNA polymerase II and III in *Acanthamoeba* (36).

Protein concentration

Protein concentration was assayed by the method of Bradford (37) using lysozyme as a standard.

SDS-polyacrylamide gel electrophoresis

Proteins from various steps in the purification process were precipitated either by the chloroform:methanol procedure of Wessel and Flugge (38) or by the addition of trichloroacetic acid to 15% (w/v) final concentration. The proteins were analyzed by denaturing polyacrylamide gel electrophoresis as described previously (36) using 0.75 mm or 1.5 mm thick gels. TIF fractions were resolved in 10%T, (2.7%C) separating gels. RNA

polymerase fractions were resolved in 12%T, (2.7%C) separating gels. Gels were visualized by staining either with Coomassie blue, or with silver by the method of Morrissey (39).

Transcription initiation factor (TIF) assays

The levels of TIF were measured by runoff transcription assays performed as described previously (40) with the following modifications. The KCl and DNA concentrations were lowered to 100 mM and 25 ng/assay respectively, in a final reaction volume of 25 μ l. Reactions were initiated by the addition of fractions containing RNA polymerase I or TIF, and incubated at 25°C for 20 min. The reactions were stopped by the addition of 225 μ l of stop buffer [50 mM sodium acetate (pH 6.0), 50 μ g/ml *E. coli* tRNA, 0.5% SDS], extracted with one volume of phenol:chloroform (1:1), one volume of chloroform:isoamyl alcohol (100:1), and precipitated with three volumes of absolute ethanol. Samples were analyzed on 8 M urea, 6%T (5%C) polyacrylamide gels, and gels were autoradiographed for 1–8 hours using Fuji RX X-ray film and DuPont Lightning-Plus intensification screens. Bands corresponding to the correct runoff transcript were excised from the gel, placed in 1 ml of deionized water, and radioactivity was estimated from Cerenkov radiation in a Beckman LS7800.

Alternatively, reactions were stopped by spotting the samples on DE81 filter discs and washing as described for the RNA polymerase assay (35). Dried discs were placed in 3 ml of deionized water and Cerenkov radiation estimated. This latter method allowed rapid determination of column fractions containing TIF activity during the purification procedure. The DE81 filter disc assay measures total RNA synthesis. Although the majority of activity is due to specific transcription, there is a contribution from non-specific and prematurely terminated/paused specific transcription. Thus to determine the specific activity at each purification step, duplicate samples were assayed, and later processed on 8 M urea polyacrylamide gels followed by autoradiography. Specific transcripts were located, excised from the gel, and quantitated by Cerenkov radiation.

DNase I footprint analysis

The plasmid pEBH10 was digested with *Eco*RI and *Hind* III. The resulting 240 bp fragment containing the wildtype *Acanthamoeba* ribosomal RNA gene promoter from -120 to +80 was used in footprinting studies as described previously (32). TIF containing fractions were incubated with the labelled DNA fragment at 25°C for 10 to 20 min, then digested with 1 μ g of DNase I for 30 sec at 25°C. For RNA polymerase I binding reactions, DNA templates were incubated with 10 μ l of TIF from the Biorex 70 step and 20 μ l of various chromatographic fractions containing RNA polymerase I, and processed as described above. Gels were electrophoresed and autoradiographed as described for TIF.

Initial determination of conditions for whole cell extraction

Ten grams of cells were homogenized in 40 ml of buffer A [20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 mM phenylmethane sulfonyl fluoride (PMSF)], containing 0.15 M KCl. One ml aliquots were removed and the salt concentration was adjusted from 0.15 M to 1.0 M KCl. The aliquots were centrifuged at $10,000 \times g$ for 10 min in an Eppendorf microfuge, the supernatants collected, and assayed for RNA polymerase activity.

To determine TIF activity, 1 ml aliquots from each extraction were diluted with buffer A to 0.2 M KCl, and loaded onto 3 ml phosphocellulose columns (previously equilibrated in buffer A containing 0.15 M KCl). The columns were washed with 1 ml of buffer A containing 0.2 M KCl, and step eluted with 6 ml of buffer A containing 0.8 M KCl at 9 ml/hr. Fractions of 2 ml were collected and 6 μ l from each fraction were assayed for TIF.

Alternatively, ten grams of cells were homogenized in 40 ml of buffer A containing 0.35 M KCl. One ml samples were removed, and centrifuged at $10,000\times g$ for 10 min. The resulting pellets were re-extracted with 1.0 ml of buffer A containing from 0.3 M to 1.0 M KCl, centrifuged at $10,000\times g$ for 10 min, and assayed for RNA polymerase. Samples were also chromatographed over 3 ml phosphocellulose columns as described above, and 6 μ l aliquots were assayed for TIF.

Conditions for polyethylenimine fractionation

Five grams of cells were homogenized with 20 ml of buffer A containing 0.35 M KCl, and centrifuged at $10,000\times g$ for 10 min. The resulting pellet was re-extracted with 10 ml of the same buffer, centrifuged as before, and the supernatants combined. To determine precipitation conditions, one ml aliquots of whole cell extracts were precipitated with increasing concentrations of PEI from 0 to 0.12% and incubated for 15 min with mixing. The aliquots were centrifuged at $10,000\times g$ for 10 min, the supernatants collected and assayed for RNA polymerase. 1.0 ml samples were diluted to 0.2 M KCl with buffer A, and chromatographed over 3 ml phosphocellulose columns as described above. Aliquots of 6 μ l were assayed for TIF.

Conditions for the elution from the PEI precipitate were determined by precipitating 40 ml of whole cell homogenate at a final concentration of 0.04% PEI. One ml aliquots were removed and centrifuged at $10,000\times g$ for 10 min. The resulting pellets were extracted with 0.5 ml of buffer A at KCl concentrations from 0 to 1.0 M, and assayed for RNA polymerase activity. To assay for TIF, 0.5 ml aliquots from the elutions were chromatographed over 3 ml phosphocellulose columns as described above.

Purification procedures

All purification steps were carried out at 4°C unless otherwise noted.

Whole cell extract. Two hundred grams of frozen *Acanthamoeba castellanii* cells were thawed by stirring in 400 ml of buffer A containing 0.4 M KCl. The cells were homogenized using a Dispax Tissuemizer until nearly all cells were disrupted (15 to 30 sec). An additional 400 ml of buffer A containing 0.4 M KCl was added and allowed to stir for 10 min. The overall KCl concentration at this step was 0.35 M. The homogenate was centrifuged in a Beckman JA 10 rotor at $10,000\times g$ for 20 min and the supernatant removed. The pellet was re-extracted with 400 ml of buffer A containing 0.35 M KCl, centrifuged as before, and the supernatants were combined. Total volume of the homogenate was 1290 ml, and the KCl concentration was adjusted to 0.35 M with 4 M KCl if necessary.

Polyethyleneimine fractionation. The homogenate was adjusted to a final concentration of 0.04% polyethyleneimine (PEI) by the dropwise addition of a 10% (v/v) solution of PEI (adjusted to pH 7.9 with HCl), and allowed to gently stir for 30 min. The precipitate was collected by centrifugation at $10,000\times g$ for 20

min. The pellet was washed by resuspending in 500 ml of buffer A containing 0.30 M KCl with a Dispax Tissuemizer, and re-centrifuged as above. To elute TIF and the RNA polymerases, the pellet was resuspended in 500 ml of buffer A containing 0.9 M KCl using the Dispax Tissuemizer. The suspension was adjusted to 0.9 M KCl, gently stirred for 15 min, and then centrifuged at $10,000\times g$ for 20 min. The supernatant was collected in a total volume of 500 ml, and contained TIF and all three classes of RNA polymerase.

Phosphocellulose chromatography. The supernatant was diluted with buffer A to 0.15 M KCl and batch loaded onto 200 ml of phosphocellulose (P11 equilibrated in buffer A containing 0.15 M KCl). The suspension was gently stirred for 1.5 hrs and the P11 collected by slowly filtering through Whatman number 4 filter paper in a Buchner filter, and was washed with one liter of buffer A containing 0.15 M KCl, resuspended in 200 ml of the same buffer, poured into a 5 cm $\times$ 30 cm column and allowed to pack. The column was washed with 5 column volumes (1 liter) of buffer A containing 0.3 M KCl. The column was developed with a 2 L linear gradient from 0.3 M to 0.9 M KCl in buffer A at 400 ml/hr. Fractions of 24 ml were collected and assayed for TIF and RNA polymerase. TIF was eluted between fractions 18–34 (approximately 430 mM KCl) in a total volume of 425 ml. RNA polymerase I was eluted between fractions 43–60 (approximately 620 mM KCl) in a total volume of 408 ml.

TIF purification

DEAE-cellulose chromatography. Phosphocellulose fractions containing TIF were pooled, diluted to 0.075 M KCl with buffer A, and batch loaded onto 100 ml of DEAE-cellulose (DE52, equilibrated in buffer A containing 0.075 M KCl). The suspension was gently stirred for 1.5 hrs and filtered as described above. The DE52 was resuspended in 100 ml of buffer A containing 0.075 M KCl and packed into a 2.5 cm $\times$ 30 cm column, and washed with 500 ml of buffer A containing 0.075 M KCl. The column was developed with a 1 liter linear gradient from 0.075 M to 0.5 M KCl in buffer A at 200 ml/hr. Fractions of 12.5 ml were collected and assayed for TIF. TIF was eluted in fractions 40–54 in a total volume of 180 ml at about 0.23 M KCl.

Heparin agarose chromatography. The pooled TIF fractions from DE52 were diluted with buffer A to a final concentration of 0.1 M KCl and were loaded onto a 2.5 cm $\times$ 30 cm column containing 25 ml of heparin agarose (equilibrated in buffer A containing 0.1 M KCl). The column was washed with 5 column volumes of buffer A containing 0.1 M KCl, and developed with a 250 ml linear gradient from 0.1 M to 0.5 M KCl in buffer A at 50 ml/hr. Fractions of 2.5 ml were collected and assayed for TIF. TIF was eluted by 0.28 M KCl, between fractions 43–64 in a total volume of 55 ml.

Matrex Green agarose chromatography. The KCl concentration of the heparin agarose TIF pool was increased to 0.3 M KCl with 4 M KCl and loaded onto a 1.5 cm $\times$ 15 cm column containing 8 ml of Matrex green agarose (equilibrated in buffer A containing 0.3 M KCl). The column was washed with 3 column volumes of buffer A containing 0.3 M KCl, followed by 5 column volumes of buffer B [10% glycerol, 20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF] containing 0.5 M KCl. The column was developed with an 80 ml linear gradient from 0.5 M to 1.8 M KCl in buffer B at 16 ml/hr.

Fractions of 1 ml were collected and assayed for TIF. TIF was eluted by 1.07 M KCl in fractions 30–54 in a total volume of 22 ml. RNA polymerase III eluted at 0.62 M KCl. The TIF pool was dialyzed against 2 liters of buffer A containing 0.15 M KCl for 5–8 hrs with one change of buffer.

Biorex 70 chromatography. The dialyzed TIF was loaded onto a 3 ml column of Biorex 70 (equilibrated in buffer A containing 0.2 M KCl). The column was washed with 5 column volumes of buffer A containing 0.2 M KCl, and TIF was eluted with a 10 ml step gradient of buffer A containing 0.5 M KCl at approximately 9 ml/hr. Fractions of 0.5 ml were collected and assayed for TIF. TIF was eluted in fractions 5–9 in a total volume of 2.5 ml, and frozen in 0.5 ml aliquots.

Glycerol gradient sedimentation. Glycerol gradients from 15% to 40% in buffer C [20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF] containing 0.1 M KCl, were formed in Beckman 13 mm×51 mm polyallomer tubes. Each gradient was layered on top with 250 μ l of pooled Biorex 70 fractions previously diluted 1:2 with buffer C containing 0.1 M KCl, then centrifuged in a Beckman SW 50.1 rotor at 45,000 rpm for 17 hrs. Gradients were fractionated into 250 μ l aliquots using an Isco density gradient fractionator and assayed for TIF. TIF sedimented between fractions 11–14, with a peak activity at fraction 12. Equivalent fractions from the 6 different gradients were pooled according to their activity, and 1–1.2 ml of each was precipitated at a final concentration of 15% trichloroacetic acid to analyze on SDS-polyacrylamide gels or to estimate protein content.

TIF-DNA glycerol gradient sedimentation. Gradient fractions containing TIF activity were pre-incubated with the pSBX60i *Ava*I/*Bam*HI derived DNA fragment at 5 μ g/ml under *in vitro* transcription conditions for 10 min, to allow formation of preinitiation complexes. The reaction mixtures were cooled at 0°C for 10 min, then diluted 1:2 with buffer C containing 0.1 M KCl. Samples of 250 μ l were layered over glycerol gradients and sedimented at 45,000 rpm for 17 hrs as above. Gradients were fractionated into 250 μ l aliquots, and assayed in the *in vitro* transcription system by the addition of RNA polymerase I, but no additional TIF or DNA.

Gel filtration. A 2.5 ml sample prepared through the Biorex 70 step was filtered through a 2.5 cm×90 cm column of Sepharose

CL-4B at 45 ml/hr, previously calibrated with blue dextran, para-nitrophenol, and standard proteins (Pharmacia). Fractions of 4 ml were collected and assayed for TIF. Standards were located by electrophoresis of samples from column fractions on SDS-polyacrylamide gels, followed by staining with silver.

RNA polymerase I purification

DEAE-cellulose chromatography. The phosphocellulose fractions containing RNA polymerase I activity were pooled, diluted to 0.075 M KCl with buffer A, and batch loaded onto 100 ml of packed DEAE cellulose (equilibrated in buffer A containing 0.075 M KCl). The suspension was stirred for 1.5 hrs, then collected by filtration as in the phosphocellulose step. The resin was slowly washed with 500 ml of buffer A containing 0.075 M KCl, then resuspended in 100 ml of the same buffer. The slurry was packed into a 2.5 cm×30 cm column, and washed with 5 column volumes of buffer A containing 0.075 M KCl. RNA polymerase I was eluted with a 1000 ml linear gradient from 0.075 to 0.5 M KCl in buffer A at 150 ml/hr. Fractions of 12.5 ml were collected and assayed for RNA polymerase I. RNA polymerase I was eluted in fractions 28–43 at 0.19 M KCl in a total volume of 154 ml.

Heparin-Sepharose chromatography. The pooled DEAE-cellulose fractions were increased to 0.3 M KCl by the addition of 4 M KCl and loaded at 50 ml/hr onto a 2.5 cm×30 cm column containing 25 ml of Heparin-Sepharose (equilibrated in buffer A containing 0.3 M KCl). The column was washed with 5 column volumes of buffer A containing 0.30 M KCl, and developed with a 250 ml linear gradient from 0.3 M to 0.8 M KCl in buffer A at 50 ml/hr. Fractions of 3 ml were collected; RNA polymerase I was eluted at 0.55 M KCl in fractions 41–57 in a total volume of 53 ml. The fractions containing RNA polymerase I activity were pooled and dialyzed against 2 liters of buffer A containing 0.025 M KCl for 5–8 hrs with one change of buffer, until the KCl concentration was less than 0.05 M.

DEAE-Sephadex chromatography. The dialyzed fractions were loaded onto a 1.5 cm×15 cm column containing 10 ml of DEAE-Sephadex (equilibrated in buffer A containing 0.05 M KCl) at 10 ml/hr. The resin was washed with 5 column volumes of buffer A containing 0.05 M KCl, and developed with a 100 ml linear gradient from 0.05 M to 0.5 M KCl in buffer A at 10 ml/hr. Fractions of 0.6 ml were collected and assayed for RNA polymerase I. RNA polymerase I was eluted at 0.11 M KCl in

Table 1. Purification of TIF

TIF fraction	Volume (ml)	Total Protein (mg)	Units/ml	Specific Activity (U/mg)	Yield (%)	Fold Purification
Whole cell extract	1,290	15,209	0.37	0.03	100	1.0
PEI eluate	500	3,115	na	na	na	na
Phosphocellulose	425	166	0.76	2.0	67.5	62
DEAE cellulose	180	39.6	0.97	4.4	36.5	140
Heparin agarose	55	16.5	2.1	7.0	24.3	221
Matrex green						
agarose	22	1.33	4.6	77	21.3	2440
Glycerol gradient	20	0.08	2.2	536	9.0	17,000

200 g of cells were used as starting material; 1 unit (U) of TIF activity is defined as the incorporation of one pmole of [³²P]-CMP into a 500 nucleotide runoff in a standard 20 min assay;
na = not assayable

fractions 19–31 in a total volume of 8.4 ml. The fractions containing RNA polymerase I were pooled and frozen in liquid nitrogen in 1 ml aliquots.

Sucrose gradient sedimentation. Using the method of Tower *et al.* (41) 5% to 20% linear sucrose gradients in buffer A containing

Figure 1. Extraction of TIF and RNA polymerases from *Acanthamoeba* cells. (A). Initial extraction of cells using increasing KCl concentrations. A whole cell extract was prepared from 10 g *Acanthamoeba* cells as described in the Experimental Procedures. The cells were homogenized with increasing concentrations of KCl, and centrifuged at $10,000 \times g$ for 10 min. Aliquots of 10 μ l were assayed for RNA polymerase. To assay for TIF, 1 ml samples of the extracts were individually chromatographed over 3 ml columns of phosphocellulose as described in the Experimental Procedures. Aliquots of 6 μ l from each fraction were assayed for TIF in the *in vitro* runoff assay. TIF (open circles), total (I+II+III) RNA polymerase (filled circles), and protein concentrations (filled triangles). (B). Second extraction of TIF and RNA polymerases using increasing KCl conditions. Ten grams of cells were homogenized at 0.35 M KCl, and 1 ml aliquots were removed and centrifuged at $10,000 \times g$ for 10 min. The resulting pellets were re-extracted with 1 ml of buffer A at increasing concentrations of KCl, then centrifuged at $10,000 \times g$ for 10 min. Aliquots of 10 μ l were assayed for RNA polymerase. Samples were then chromatographed over phosphocellulose as described in the Experimental Procedures, and 6 μ l aliquots were assayed for TIF. TIF (open circles), total RNA polymerase (filled circles), and protein concentrations (filled triangles).

0.5 M KCl were formed in Beckman 25 mm $\times$ 89 mm polyallomer quick seal tubes. One ml aliquots of pooled polymerase fractions were layered on top of each gradient, followed by centrifugation at 50,000 rpm, 2°C for 17 hrs in a Beckman Vti 50 vertical rotor. The gradients were fractionated at 4°C into 1 ml aliquots, assayed for RNA polymerase activity, then stored in liquid nitrogen. RNA polymerase I sedimented between fractions 6 and 10 in a total volume of 5 ml per gradient. Fractions were later assayed using the promoter runoff RNA transcription system.

RESULTS

TIF was purified based on its ability to direct accurate initiation of transcription from a cloned rDNA template when supplemented with RNA polymerase I. Using conventional chromatography and glycerol gradient ultracentrifugation, TIF has been purified approximately 17,000 fold. The overall purification of TIF is shown in Table I. From the same whole cell extracts, RNA polymerase I was purified to apparent homogeneity with a yield of 8.1% and a specific activity of 315 U/mg. Table II shows the overall purification for RNA polymerase I.

Preparation of whole cell extracts

Previously, TIF was fractionated from S100 extracts prepared by the method of Weil *et al.* (42) and shown to be capable of directing specific transcription from *Acanthamoeba* ribosomal DNA templates *in vitro* (40). The processing of large quantities of cells for these extracts was hindered by the limited capacity for high speed centrifugation. As an alternative approach, whole cell extracts were utilized which allowed for low speed centrifugation of large volumes (43).

Conditions for optimal extraction of TIF and RNA polymerases from *Acanthamoeba* cells were determined by homogenizing cells at increasing concentrations of KCl from 0.1 to 1.0 M. RNA polymerase activity was measured directly from the extracts, and maximum levels were obtained by 0.35 M KCl (see Figure 1A). RNA polymerase I contributed 60% of the total polymerase activity (data not shown). Higher KCl concentrations resulted in no additional extraction of RNA polymerase. As the concentration of KCl was increased above 0.35 M, total RNA polymerase activity measured in the crude extract actually declined due to increased solubilization of inhibitory components.

TIF levels in the crude extract could not be consistently determined in the runoff transcription assay due to the presence of interfering components associated with the preparation of

Table 2. Purification of RNA polymerase I

RNA Pol I fraction	Volume (ml)	Total Protein (mg)	Units/ml	Specific Activity (U/mg)	Yield (%)	Fold Purification
Whole cell extract	1,290	15,209	4.36	0.37	100	1.0
PEI eluate	500	3,115	10.1	1.62	89.8	4.4
Phosphocellulose	408	184	85.9	13.1	42.9	35
DEAE cellulose	154	69.8	13.3	29.3	36.4	79
Heparin						
Sepharose	53	23.1	21.1	48.1	19.8	130
DEAE Sephadex	8.4	8.5	73.2	72.4	10.9	196
Sucrose gradient	35	1.46	13.1	315	8.1	852

200 g of cells were used as starting material. One unit (U) of activity is equal to 1 nmol UMP incorporated in 10 min in the standard (non-specific) assay.

whole cell extracts. To determine TIF activity, 1 ml aliquots of cellular homogenates were chromatographed over 3 ml phosphocellulose columns as described in the Experimental Procedures. These fractions were sufficiently free of inhibitory substances to allow accurate assay of TIF. Maximum TIF activity was recovered from cells extracted at 0.6 M KCl and higher (Figure 1A). However, this KCl concentration created difficulties in subsequent steps by requiring excessive dilution or dialysis of large volumes.

As an alternative to the high salt extraction, cells were extracted utilizing an initial KCl concentration of 0.35 M, followed by re-extraction of the resultant pellet. Secondary extractions carried out with 0.15 M to 1.0 M KCl resulted in a relatively uniform recovery of TIF at 0.35 M and above, and a constant recovery of RNA polymerase (see Figure 1B). The supernatant arising from the second extraction also contained significant RNA polymerase activity, which may have been due to increased solubilization of protein, or the release of RNA polymerase trapped within the cellular pellet. Similar results have been observed in the purification of yeast DNA-dependent RNA polymerases, where subsequent extraction of an S35 pellet increased recovery of total RNA polymerase activity by approximately 10% (44). Thus two successive extractions carried

out at 0.35 M KCl resulted in maximal recovery of RNA polymerase and TIF while keeping the total salt concentration fairly low.

Polyethyleneimine fractionation

Polyethyleneimine precipitation. Conditions for precipitation and elution of RNA polymerase I and TIF with polyethyleneimine (PEI) were determined as described by Spindler *et al.* (43). Whole cell extracts were prepared as described above, and 1 ml aliquots were precipitated with increasing amounts of PEI from 0 to 0.12% (v/v). All RNA polymerase was precipitated by 0.04% PEI (see Figure 2A). The observed loss of activity at 0.04% PEI

Figure 2. Fractionation of whole cell extracts with polyethyleneimine (PEI). (A). Precipitation of TIF and RNA polymerases by PEI. Whole cell extracts were prepared from 5 g of cells as described in Experimental Procedures, and 1 ml samples were removed and precipitated with increasing amounts of PEI from 0 to 0.12% final concentration (v/v). After centrifugation at $10,000\times g$ for 10 min, the supernatants were transferred and 10 μ l aliquots were assayed for RNA polymerase. Samples were then fractionated over phosphocellulose, and 6 μ l were assayed for TIF. TIF (open circles), total RNA polymerase (filled circles), and protein concentrations (filled triangles). (B). Elution of TIF and RNA polymerases from PEI precipitates. 40 ml of whole cell extract was prepared as described in Figure 1, and PEI was added to a final concentration of 0.04%. One ml aliquots were removed, and centrifuged at $10,000\times g$ for 10 min. The resulting precipitates were extracted with increasing concentrations of KCl as described in the Experimental Procedures. After centrifugation at $10,000\times g$ for 10 min, 10 μ l aliquots were assayed for RNA polymerase. One ml samples were also fractionated over phosphocellulose, and 6 μ l aliquots were assayed for TIF. TIF (open circles), total RNA polymerase (filled circles), and protein concentrations (filled triangles).

Figure 3. Phosphocellulose chromatography. (A). A 200 ml phosphocellulose column loaded with the PEI eluate was processed with a 2 liter linear gradient from 0.3 M to 0.9 M KCl in buffer A. Fractions were assayed for TIF (open circles) and RNA polymerase (filled circles). For RNA polymerase reactions, 10 μ l from each fraction were included. TIF reactions contained 8 μ l from each fraction. (B). KCl gradient (broken line) and protein profile (A₂₈₀; solid line) from phosphocellulose column chromatography. (C). Comparison of TIF activity determined by the DEAE filter disc assay or the gel assay as described in the Experimental Procedures. Transcription assays were performed with a 2-fold increase in volume from normal. Upon terminating the reaction, half of the sample (25 μ l) was spotted onto a DEAE filter disc and processed as described in the Experimental Procedures, and the other half was processed for analysis by urea-polyacrylamide gel electrophoresis. The activity profiles for the gel assay (open circles) and the DEAE filter disc assay (filled circles) are shown.

may have resulted from inhibition by excessive PEI levels. To examine this possibility, the supernatants were chromatographed over phosphocellulose columns as described above, which effectively removes PEI (2,4). This step did not change the activity profile.

TIF in the PEI-treated extracts could not be directly assayed in the *in vitro* transcription system due to the presence of inhibitory elements and/or excessive PEI. Thus, aliquots chromatographed on phosphocellulose as described above were assayed for TIF. All TIF activity was precipitated at a final PEI concentration of 0.025% (Figure 2A).

Elution from polyethyleneimine precipitate. Conditions for the elution of TIF and RNA polymerase following PEI precipitation were determined by precipitating whole cell homogenates at a final concentration of 0.04% PEI. The resulting precipitates were extracted at increasing ionic strength. Maximum RNA polymerase activity was eluted from the PEI precipitate at 0.8 M KCl, while maximum TIF activity was obtained at 0.9 M KCl (Figure 2B).

The PEI precipitation and elution conditions determined for optimal recovery of RNA polymerase or TIF differed slightly. The higher PEI and KCl concentrations were used so that all proteins could be obtained from the same initial extract. The conditions determined in these small volumes were reproducible when the preparation was scaled up to 200 g of cells.

All purification steps are discussed in detail in the Experimental Procedures. Conditions for the preparation of whole cell extracts, and PEI fractionation steps were reproducible and thus fractions at these steps were not routinely assayed. 200 g of cells were extracted twice at 0.35 M KCl, then precipitated at 0.04% PEI, and the proteins of interest were eluted at 0.9 M KCl.

Phosphocellulose chromatography

The PEI eluate was diluted to 0.15 M KCl and batch loaded onto phosphocellulose (P11), packed into a column and developed with a linear KCl gradient. Two distinct RNA polymerase peaks were observed at approximately 0.43 M and 0.62 M KCl (see Figure 3A and 3B). These peaks corresponded to RNA

polymerase III and I respectively as determined by their sensitivity to α -amanitin inhibition (36). RNA polymerase I containing fractions were pooled, and were essentially free of RNA polymerase II and III activities (data not shown). The bulk of RNA polymerase II was eluted during the initial loading and washing of the resin.

TIF was found to elute at approximately 0.43 M KCl, which coincided with the RNA polymerase III peak (Figure 3A and 3B). To expedite the assay for TIF at this and subsequent purification steps, a DEAE filter disc assay similar to that designed by Sawadogo and Roeder (45) was used (see Experimental Procedures). When compared to the gel electrophoresis assay, the DEAE filter disc assay did not give equivalent levels of radioactive nucleotide incorporation as measured by Cerenkov radiation. However, coincident activity profiles were produced thus allowing accurate determination of those fractions containing TIF during each chromatographic step. An example is shown for the profile obtained from phosphocellulose chromatography (Figure 3C).

At this step, RNA polymerase I was purified approximately 35-fold from the WCE step with a total yield of 43%, and TIF was purified approximately 62-fold with a yield of 68%. Purification of TIF and RNA polymerase I following separation by phosphocellulose chromatography were performed independently.

Purification of TIF

DEAE cellulose chromatography. The fractions containing TIF from the phosphocellulose step were chromatographed over DEAE cellulose (DE52), and TIF was eluted at approximately

Figure 4. Estimation of native molecular weight of TIF. (A). Gel Filtration. A 2.5 ml sample from the Biorex 70 concentration step was run through a 2.5 cm $\times$ 90 cm column of Sepharose CL-4B at 40 ml/hr. Position of TIF elution is shown by the arrow. The protein standards used are (thy) thyroglobulin, (fer) ferritin, (cat) catalase, (hem) hemoglobin, (cyt) cytochrome c. (B). Glycerol Gradient Ultracentrifugation. TIF was sedimented in a glycerol gradient as described in the Experimental Procedures. Fractions 1 and 20 correspond to the top and bottom of the gradient respectively. Duplicate gradients were run containing proteins of known molecular weights as standards. TIF reactions contained 10 μ l from each fraction. The protein standards used are (fer) ferritin, (cat) catalase, and (BSA) bovine serum albumin.

Figure 5. DNase I protection of coding and non-coding (RNA-like) strands of rDNA by TIF. Fractions derived from various chromatographic steps were assayed for the ability to protect the rDNA template. 20 μ l samples from various chromatographic steps were preincubated with a 240 bp rDNA fragment for 10 min prior to digestion with DNase I. (A) Coding strand (B) Non-coding strand. Lanes: (1) no TIF; (2) Phosphocellulose; (3) DEAE-cellulose; (4) heparin agarose; (5) Matrex green agarose; (6) Biorex 70.

0.23 M KCl (data not shown). This step resulted in a 2-fold purification of TIF, with an overall yield of 37%.

Heparin agarose chromatography. The DEAE cellulose fractions containing TIF were chromatographed over a heparin agarose column developed with a linear KCl gradient. TIF was eluted at approximately 0.28 M KCl (data not shown). This step resulted in another 2-fold purification of TIF with a total yield of 24%. Throughout these steps, RNA polymerase III co-chromatographed with TIF.

Matrex green agarose chromatography. The fractions containing TIF from the heparin agarose step were chromatographed over a Matrex green agarose column, which was developed with a linear KCl gradient in buffer B (see below). TIF eluted at about 1.07 M KCl and RNA polymerase III eluted at 0.62 M KCl. In this step, TIF was purified approximately 11-fold with an overall yield of 21%. Matrex green agarose was effective at separating RNA polymerase III from TIF when the elution buffer (buffer B) contained a glycerol concentration of 10% (v/v), but RNA polymerase III eluted at a high KCl concentration similar to TIF if chromatography was carried out in buffer A (20% glycerol).

The net purification to this stage was 2,400-fold over the crude extract, with a yield of 21%. To further purify and attempt to partially characterize TIF, the fractions containing TIF activity were concentrated by chromatography over Biorex 70 as described in Experimental Procedures and the concentrated fractions were sedimented through glycerol gradients.

Glycerol Gradient Ultracentrifugation. Sedimentation through 15% to 40% glycerol gradients resulted in a 7-fold purification. Table I summarizes the purification of TIF to this step. Duplicate gradients were also run containing standard proteins, and TIF was found to sediment between fractions 11 and 14 with a peak activity at fraction 12. This corresponded to a sedimentation coefficient of 12.33 S (Figure 4B).

Figure 6. Accurate transcription of rDNA *in vitro* with purified RNA polymerase I. Sucrose gradient fractions were assayed for TIF/promoter-dependent RNA polymerase I activity in the *in vitro* transcription system to determine its ability to specifically initiate transcription. The reactions each contained 1 μ l of TIF from a Biorex 70 pool, and 1 μ l from individual sucrose gradient fractions. Reactions were electrophoresed in denaturing gels and autoradiographed as described in the Experimental Procedures. Specific transcripts were excised from the gels, and estimated by Cerenkov radiation. Promoter-dependent RNA polymerase I activity (open circles) and bulk RNA polymerase I activity assayed using calf thymus DNA (filled circles).

Gel filtration. A sample of TIF prepared through the Biorex 70 step was chromatographed through Sepharose CL-4B. The column was previously standardized with a high molecular weight gel filtration calibration kit (Pharmacia). TIF was found to elute after ferritin and just before catalase with a Stoke's radius of 57.1 Å (Figure 4A). The molecular weight of TIF was calculated from the Stoke's radius and sedimentation coefficient according to Siegel and Monty (46), and found to be 289,000.

Footprinting of TIF

DNase I protection studies previously have demonstrated that TIF stably binds to the region from approximately -69 to -12 on the *Acanthamoeba* rRNA gene promoter (32). To determine at what points in the purification process TIF could produce distinct footprints, various chromatographic fractions were assayed for protection against DNase I digestion. Fractions containing peak transcriptional activity from each column were assayed for footprinting activity (Figure 5). Protection against DNase I digestion was observed as early as the DEAE cellulose step (see lane 3), and in all subsequent chromatographic steps. TIF was thus either sufficiently pure or concentrated in peak fractions to protect the rDNA promoter. Fractions from whole cell extracts and PEI eluates were unable to produce distinct footprints on the *Acanthamoeba* rDNA promoter, and thus no correlation between specific DNA binding and transcriptional activation could be demonstrated at these early steps.

The pools from each chromatographic step gave undistinguished or very weak footprints (data not shown). This is likely due to dilution of TIF during pooling of the active chromatographic fractions, since 1 to 2 units of TIF per ng of template are required to generate protection against DNase I.

Purification of RNA polymerase I

Purification of *Acanthamoeba* RNA polymerase I has been previously reported by Spindler et al. (43) from low salt whole

Figure 7. SDS-polyacrylamide gel electrophoresis of sucrose gradient purified RNA polymerase I. Numbers at the top of the figure correspond to fraction numbers from Figure 6. Letters a-h represent the subunits of RNA polymerase I determined in this study. The 35 kD subunit described by Spindler et al. (43) was not found. The two small subunits (10 kD) of the enzyme, are barely visible below polypeptide h.

cell extracts (0.025 M KCl). By extracting cells at higher salt concentrations, and using modifications of the original purification procedure, final recovery of RNA polymerase has been increased nearly 10-fold from an equivalent number of cells.

DEAE cellulose chromatography. The fractions from phosphocellulose chromatography containing RNA polymerase I were pooled and chromatographed over a DEAE cellulose (DE52) column. RNA polymerase I eluted at about 0.19 M KCl (data not shown). At this step RNA polymerase I was purified an additional 2-fold with an overall yield of 36%.

Heparin-Sepharose Chromatography. The DEAE cellulose fractions were chromatographed over Heparin-Sepharose, which was developed with a linear KCl gradient from 0.3 to 0.8 M. RNA polymerase I was eluted at about 0.55 M KCl. This step resulted in another 2-fold purification and overall yield of 20%. The fractions from this step were pooled and dialyzed.

DEAE-Sephadex Chromatography. The dialyzed fractions were chromatographed over a DEAE-Sephadex column, which was developed with a linear gradient. RNA polymerase I eluted at about 0.11 M KCl. In this step, RNA polymerase I was purified 1.5 fold, with an overall yield of 11%. The overall purification to this step was 196-fold.

The three chromatographic steps on DEAE cellulose, Heparin-Sepharose, and DEAE-Sephadex, eliminated 95% of the protein. These steps increased purification only 5-fold due to the low yields, but decreased protein content 22-fold.

Figure 8. DNase I footprinting of coding and non-coding strands of *Acanthamoeba* rDNA by RNA polymerase I. Peak fractions from different chromatographic steps were assayed for the ability to protect the rDNA template from DNase I digestion. The rDNA template was incubated with 10 μ l of TIF purified through the Biorex 70 step, and 30 to 50 milliunits of RNA polymerase I purified through various chromatographic steps. (A) Coding strand (B) Non-coding (RNA-like) strand. Lanes: (1) No protein; (2) TIF alone; (3) Phosphocellulose; (4) DEAE cellulose; (5) Heparin-Sepharose; (6) DEAE-Sephadex; (7) Sucrose gradient.

Sucrose Gradient Sedimentation. The DEAE-Sephadex fractions were sedimented through 5% to 20% sucrose gradients. The gradients were fractionated and assayed for RNA polymerase activity, and for specific transcriptional activity in the promoter runoff assay. RNA polymerase I was sedimented between fractions 6 and 12, with a peak at fraction 8 (see Figure 6). RNA polymerase I prepared from this procedure was used in the reconstituted *in vitro* transcription system, and was capable of mediating accurate transcription of rRNA genes (Figure 6). Comparison of the two profiles demonstrate that the two activities overlap, but did not exactly coincide. The peak in RNA polymerase I activity assayed by the *in vitro* promoter-dependent transcription system was found to sediment two fractions further in the gradients than the peak activity assayed by the non-specific assay. These results are similar to those observed in mouse (41) and can be interpreted as a difference between RNA polymerase I that is transcriptionally active or inactive on rDNA promoters (34,47–49).

Sucrose gradient sedimentation increased purification to 852-fold over the crude homogenate, with a specific activity of 315. From each one ml fraction, 150 μ l samples were precipitated and 20% of the sample run on 12% SDS-polyacrylamide gels. Sucrose gradient fractions analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis displayed 8 bands whose M_R = 185, 133, 41.5, 39, 22.5, 17.5, 15.5, 13.3 kD (see Figure 7). These polypeptides correspond to subunits of RNA polymerase I identified earlier by (43,50), except the 10 kD subunit(s) are not well resolved. In contrast to these earlier results, one subunit was not observed on denaturing gels. Absent was the 35 kD subunit which may be a degradation product of the 41.5 kD subunit (4) or a modified form of the 39 kD subunit, and thus not a true subunit of RNA polymerase I. The 39 kD subunit is not significantly modified into the form found in inactive RNA polymerase I from encysted cells, previously identified as a 37 kD subunit (50) because of its altered mobility in SDS polyacrylamide gel electrophoresis (Kubaska and Paule, unpublished).

Footprinting of RNA polymerase I

Peak fractions from the different chromatographic and sedimentation steps were assayed for their ability to stably associate with the TIF-rDNA complex by DNase I protection studies. The homogeneous RNA polymerase I obtained from sucrose gradient sedimentation retained the capacity to extend the TIF footprint from -12 to approximately $+20$ (Figure 8). Previously, DNase I footprinting experiments showed that RNA polymerase I was able to extend the TIF-rDNA protected region from -14 to approximately $+20$ on the non-coding strand (32,34). Fractions from early chromatographed steps were also able to demonstrate footprints. Peak fractions from as early as the phosphocellulose step were able to produce visible footprints. The stable binding of RNA polymerase I was dependent on the presence of TIF as previously shown (32) (data not shown).

DISCUSSION

The purification procedure presented here allows isolation of both *trans*-acting components required for specific transcription of *Acanthamoeba* rRNA *in vitro*. RNA polymerase I was purified based on nonspecific transcription of calf thymus DNA *in vitro*. It has been purified to apparent homogeneity with a total yield of 1.46 mg from 200 g of cells, a quantity 10 fold greater than

previously (43), but with a comparable specific activity of 315. RNA polymerase I previously purified from *Acanthamoeba* by Spindler et al. (43) consisted of 10–11 polypeptides (M^R =185, 133, 41.5, 39, 37, 35, 22.5, 17.5, 15.5, 13.3, 10 kD). The polymerase purified by the procedure described here does not have the 37 kD or 35 kD polypeptides (Figure 7). The 35 kD subunit previously identified may have been a degradation product of a larger subunit. Alternatively, it may have been a modified form of the 39 kD subunit. The 37 kD 'subunit' has been shown to be a modified form of the 39 kD subunit (Kubaska and Paule, unpublished) whose appearance correlates with the growth-rate-dependent shutoff of rRNA transcription. The 39 kD subunit is present in a stoichiometry of 2 in both RNA polymerase I and III (43,51), and based upon homology to the *Saccharomyces* polymerase, is a homolog of the *E. coli* α -subunit (52). Also, in the same yeast it has recently been shown that there are two subunits with identical molecular weights of 10 kD in SDS-polyacrylamide gels, both of which are common to all three RNA polymerases (Treich, Carles, Riva and Sentenac, personal communication). Thus, we surmise that RNA polymerase I from *Acanthamoeba* consists of 12 subunits [185₁ 133₁ 41.5₁ 39₂ 22.5₂ 17.5₁ 15.5₁ 13.3₁ 10 α_1 10 β_1].

The TIF fraction was purified based on its ability to direct accurate initiation of transcription *in vitro* using rDNA templates and RNA polymerase I. It has been purified approximately 17,000-fold, with a yield of 9% and a specific activity of 536. The properties exhibited by TIF parallel those of the components originally identified as fraction D in human and mouse extracts (53,54). Of the four phosphocellulose chromatographic fractions initially identified by Mishima et al. (53), two of these fractions have been identified as an RNase inhibitor (24), and RNA polymerase I respectively (24,55). Fraction D has been demonstrated to be the species-specific factor (56) required for transcription *in vitro*, and involved in stable complex formation (54). Stable binding to specific rDNA core promoter sequences has since been shown by DNase I protection studies (25,32). In *Acanthamoeba*, the chromatographic properties of TIF and RNA polymerase I on phosphocellulose are reversed in comparison to their presumed mammalian counterparts, Factor D and Factor C, respectively (53). Despite this difference, these proteins appear to function in an analogous manner.

A *trans*-acting factor that binds to mammalian and *Xenopus* rDNA promoters has been purified to homogeneity and cloned. UBF binds to the core promoter and upstream control element in human rDNA (22,25) and appears to act as an assembly factor for TIF-IB (or SL1) (57,58). The latter appears to be the species-specific factor indispensable for transcription. In concert, TIF-IB and UBF appear to function in a manner similar to factor D in mouse, and the TIF fraction in *Acanthamoeba* [reviewed in (6)].

In the *Acanthamoeba* system, TIF appears to be the only required factor fraction besides RNA polymerase I for transcription *in vitro*. This does not exclude the possibility that other transcription factors may exist that co-purify with TIF or RNA polymerase I or whose effects are observed only *in vivo*.

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