Isolation of multiple protein factors involved in ribosomal DNA transcription

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ABSTRACT

Studies were made of the molecular mechanisms which regulate ribosomal gene transcription in response to changes in the growth rate of cells. Extracts prepared from exponentially growing Ehrlich ascites cells faithfully and efficiently transcribe cloned mouse rDNA, whereas extracts from growth-arrested cells are virtually inactive. In an attempt to identify and characterize functionally the proteins that mediate the accuracy and the control of transcription initiation, a fractionation procedure was developed which allows the purification of RNA polymerase I and four accessory factors that are required for transcription initiation at the ribosomal gene promoter. Starting from about 300 ml of cell extract, each of the individual factors and the polymerase was purified on at least four different chromatographic columns, including ion-exchange chromatography on DEAE-Sepharose, heparin-Ultrogel, Mono Q and Mono S, gel filtration and specific affinity chromatography. The resulting protein fractions are functionally active, as shown by reconstitution of specific rDNA transcription in the presence of purified polymerase and the additional factors.

INTRODUCTION

During the last few years, detailed analysis of many eukaryotic genes has revealed at least two types of *cis*-acting regulatory elements: promoters and enhancers. These elements are recognized by *trans*-acting proteins via either protein–DNA or protein–protein interactions. The primary goal of current research is to identify and characterize these proteins and to determine their precise role in cellular physiology. A useful model for such studies is provided by the ribosomal RNA genes, which belong to the most actively transcribed genes in growing cells. Further, rDNA transcription is regulated in response to a variety of extracellular stimuli including growth factors, steroid and peptide hormones and nutrient supply [1–4], and therefore represent an excellent experimental system to study the molecular mechanisms which couple gene expression and cell proliferation.

A major step towards understanding rDNA transcription regulation at the molecular level was the development of cell-free systems which faithfully transcribe cloned rDNA sequences [5]. The avail-ability of such *in vitro* systems facilitates the identification and functional analysis of both the *cis*-acting DNA sequences and the *trans*-acting proteins involved in rDNA transcription initiation. To our knowledge, rDNA transcription initiation is mediated by a complex interplay between RNA polymerase I (pol I) and at least four additional transcription initiation factors, termed TIF-IA, TIF-IB, TIF-IC and UBF [6].

TIF-IB and UBF are specific DNA-binding proteins which form a strong cooperative complex on the rDNA gene promoter. The binding of TIF-IB (the protein that confers promoter specifity to pol I) to the rDNA promoter is an early step in the formation of an active transcription complex [7,8]. Binding of TIP-IB and UBF to the rDNA promoter enables the polymerase and also the two accessory factors TIF-IA and TIF-IC to assemble into a productive initiation complex. Both TIF-IA and TIF-IC do not bind to DNA but rather associate with pol I. Whereas TIF-IC may serve a function in selection of the correct start site [9], TIF-IA is a positive-acting factor whose level or activity fluctuates in response to the physiological state of cells [4,10].

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma (Deisenhofen, Germany).

DEAE-Sepharose CL-6B, CM-Sephadex, heparin-Ultrogel A4/R, Q-Sepharose and the prepacked fast protein liquid chromatographic (FPLC) columns Mono Q HR 5/5, Mono S HR 5/5, S-Sepharose High Performance 16/100 and HiLoad 26/60 Superdex 200 were obtained from Pharmacia–LKB (Freiburg, Germany). A polyethyleneimine (PEI) high-performance liquid chromatographic (HPLC) column was obtained from Baker (Gross-Gerau, Germany).

Cell culture and extract preparations

Ehrlich ascites cells were cultured in RPMI medium containing 5% newborn calf serum for 20–40 h. Transcriptionally active extracts were obtained from a logarithmically growing cell culture ($9 \cdot 10^5$ cells/ml). S 100 extracts were prepared according to Weil et al. [11] and nuclear extracts according to Dignam et al. [12].

In vitro transcription assays

The soluble cell-free transcription system and the analysis of the RNA synthesized has been described previously [5]. Usually, 50–100 ng of plasmid pMr600 truncated with Eco RI were incubated with 15 μl of a mixture of S-100 and nuclear extracts in a total volume of 25 μl containing 12 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.9), 0.1 mM EDTA, 0.5 mM dithioerythriol (DTE), 5 mM MgCl$_2$, 75 mM KCl, 10 mM creatine phosphate, 12% (v/v) glycerol, 0.66 mM each of ATP, CTP and UTP, 0.01 mM GTP and 1.5 μCi of [α-^{32}P]GTP (400 Ci/mmol). After incubation for 60 min at 30°C, the nucleic acids were extracted, precipitated and analysed on non-denaturing 5% polyacrylamide gels.

Total pol I activity was assayed in a 25-μl reaction mixture containing 6 mM Tris–HCl (pH 7.9), 0.1 mM EDTA, 5 mM MgCl$_2$, 80 mM KCl, 6% glycerol, 0.5 mM DTE, 0.66 mM each of ATP, CTP and GTP, 3.6 μM [^{3}H]UTP (5.5 Ci/mmol), 7.5 μg of calf thymus DNA and 5 μg of α-amanitin. After incubation for 30 min at 30°C, the reaction was stopped by the addition of 0.2 ml of saturated Na$_4$P$_2$O$_7$ containing 50 μg of carrier DNA and precipitated with 5% trichloroacetic acid. The precipitates were collected on glass-fibre filters and quantified by scintillation counting.

One unit of RNA polymerase I activity represents the incorporation of 1 pmol of [^{3}H]UMP during 10 min of incubation under these conditions.

Purification of transcription factors

A typical factor preparation was started from 300 ml (1.8 g of protein) of a mixture of nuclear and cytoplasmatic extracts prepared from cultured Ehrlich ascites cells. All buffers contained 0.5 mM DTE and 0.5 mM phenylmethylsulphonylfluoride (PMSF), added immediately prior to use. Extract proteins were applied to a DEAE-Sepharose CL-6B column, washed with buffer A [20 mM Tris–HCl (pH 7.9)–0.1 mM EDTA–20% glycerol] containing 100 mM KCl and step eluted at 280 mM KCl. This fraction, which contains pol I and the four transcription factors, was loaded (at 200 mM KCl) on a heparin-Ultrogel A4-R column and eluted with buffer AM (buffer A with 5 mM MgCl$_2$) containing 400, 600 and 1000 mM KCl, yielding fractions H-200 (flowthrough fraction), H-400, H-600 and H-1000, respectively.

TIF-IA and TIF-IC activities present in the H-200 fraction were concentrated on a Q-Sepharose column. Separation of TIF-IA and TIF-IC was achieved by HPLC on a polyethylenimine column using a linear gradient from 0.1 to 1 M KCl. TIF-IC and TIF-IA eluted at 300 and 700 mM KCl, respectively. As a next step TIF-IA, was applied to a Mono Q column and eluted at 230 mM KCl in a gradient from 200 to 300 mM KCl. TIF IC was purified to near homogeneity on an Orange A dye affinity matrix from which it elutes at 230 mM KCl.

RNA polymerase I present in the H-400 fraction was further purified on S-Sepharose High Performance, HiLoad 26/60 and Mono Q HR5/5 columns. TIF-IB was recovered in the H-600 fraction and purified further by chromatography on CM-Sepharose, Mono S (FPLC) and affinity chromatography on calf thymus DNA cellulose [8].

UBF activity was present in the H-1000 fraction and purified to homogeneity by chromatography on a Mono Q FPLC column followed by a specific oligonucleotide affinity column [13].

Conventional chromatography

In the first step of the purification procedure 300 ml of cell extracts (1.8 g of protein) were loaded on a DEAE-Sepharose CL 6B column (80 ml), equilibrated with buffer A at a flow-rate of 3 ml/min; the fraction size was 8 ml.

Chromatography on heparin-Ultrogen A4/R, CM-Sephadex and Q-Sepharose was performed in buffer AM 100 at the appropriate flow-rates; fraction sizes were normally one tenth of the column volume. Protein absorbance was monitored at 280 nm (Uvicord SD). The individual column fractions were dialysed against buffer AM-100 before they were assayed in the reconstituted transcription system.

High-performance liquid chromatography

An HPLC system manufactured by Pharmacia–LKB was used. Buffers were degassed and filtered through a 0.22-μm membrane (Sartorius) prior to use. Ion-exchange HPLC was performed on a PEI column (4 ml) using AM buffer with a KCl gradient from 0.1 to 1 M. The flow-rate was 0.5 ml/min and the fraction size was 1.0 ml.

Fast protein liquid chromatography

An FPLC system (Pharmacia) consisting of an LCC-Controller-500 Plus (gradient programmer), two P-500 pumps, a UV-M monitor (280 nm), an REC 102 recorder, an MV-7 multi-position motor valve and a FRAC 100 fraction collector was used. For all columns buffer AM [20 mM HEPES (pH 7.9)–0.1 mM EDTA–5 mM MgCl$_2$–20% glycerol] was used. All buffers were degassed and ultrafiltered (Sartorius 0.22-μm filter). Ion-exchange FPLC was performed with Mono Q HR 5/5 and Mono S HR 5/5 columns at a flow-rate of 0.5 ml/min, fraction size 0.5 ml and pressure 2 MPa. Cation-exchange FPLC was carried out on an S-Sepharose High-Performance column at a flow-rate of 2 ml/min, pressure 0.3 MPa and fraction size 4 ml. Gel filtration FPLC was performed with HiLoad 26/60 (Superdex 200) at a flow-rate of 2 ml/min and a pressure of 0.3 MPa; 3-ml fractions were collected.

DNA affinity chromatography

UBF was purified to apparent homogeneity on a sequence-specific DNA affinity column as described by Bell and co-workers [14,15]. Affinity chromatography on calf-thymus DNA cellulose was carried out according to Schnapp et al. [8].

Protein analysis

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [16]. Silver staining was carried out according to the method of Blum et al. [17]. Protein was determined by the method of Bradford [18].

RESULTS AND DISCUSSION

Four factors besides RNA polymerase I are required for mouse rDNA transcription initiation

To study the mechanisms directing faithful transcription initiation of the ribosomal genes, the purification of the various factors involved in this process is required. We have shown previously that rDNA transcription in vitro reflects the rRNA synthetic capacity of the cells from which the extracts have been prepared. Extracts derived from cultured cells support transcription of cloned rDNA whereas extracts from tissues or slowly growing cells are virtually inactive [4,10]. This finding implies that the activity of one or several proteins involved in ribosomal gene transcription is growth controlled and, therefore, the individual proteins should be prepared from cultured cells. The concentration of the rDNA transcription initiation factors within the cell is known to be extremely low and it is laborious and expensive to obtain enough cultured cells as starting material. For this reason it was necessary to develop a fractionation procedure which allows the purification of both the polymerase and the additional essential factors from the same starting material. The procedure established involves a combination of several chromatographic steps, including ion-exchange chromatography on DEAE-Sepharose, Mono Q and Mono S (FPLC), gel filtration and specific affinity chromatography.

Using the fractionation strategy shown in Fig. 1A, we isolated four factors (termed TIF-IA, TIF-IB, TIF-IC and UBF) that in addition to pol I are required for efficient transcription initiation from the mouse rDNA promoter. The complete reconstituted system contains five fractions including RNA polymerase I and four auxiliary factors. The re-
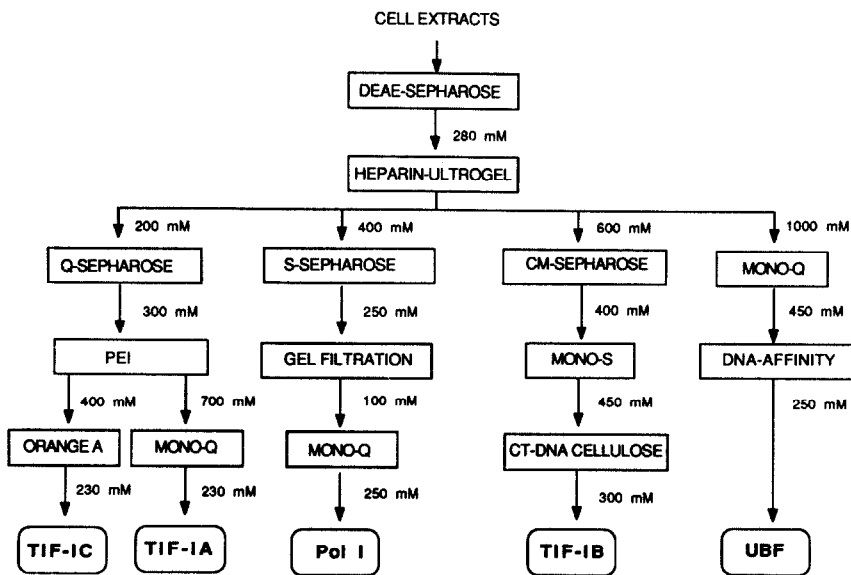
quirement of the various factors for transcription initiation from the mouse rDNA promotor is shown in Fig. 1B. When all fractions are present at optium concentrations 297 nucleotide (nt) run-off transcripts are synthesized (lane 1). In the absence of pol I or any of the other factors, no specific transcripts are generated (lanes 2–6), indicating that each of these factors plays an indispensable role in transcription initiation. The labelled bands on the top of the gel represent non-specific transcripts. Importantly, although most of the factor preparations used are not yet homogeneous, they show no cross-contamination, as the full complement of all four factors was necessary for efficient transcription.

Purification of RNA polymerase I

We shall focus here on the isolation of pol I, but all purification problems and working conditions can be transferred to the four essential pol I specific transcription initiation factors. The purification of these factors is described under Experimental. Eukaryotic RNA polymerase I is notoriously unstable and thermolabile and therefore all operations were carried out at 4°C. To stabilize the enzyme, 20% glycerol is included in all buffers, which results in low flow-rates and high back-pressures for Mono Q and Mono S FPLC columns (flow-rate 0.5 ml/min, pressure 2 MPa). In addition, dilution of the enzyme preparations frequently resulted in severe losses of activity. During all chromatographic steps steep salt gradients resulted in greater recoveries of activity than did shallow gradients, although the resolution and the degree of purification were better in the latter instance.

A

CELL EXTRACTS

DEAE-SEPHAROSE

280 mM

HEPARIN-ULTROGEL

| 200 mM | 400 mM | 600 mM | 1000 mM |

| Q-SEPHAROSE | S-SEPHAROSE | CM-SEPHAROSE | MONO-Q |

| 300 mM | 250 mM | 400 mM | 450 mM |

| PEI | GEL FILTRATION | MONO-S | DNA-AFFINITY |

| 400 mM 700 mM | 100 mM | 450 mM | 250 mM |

| ORANGE A | MONO-Q | MONO-Q | CT-DNA CELLULOSE |

| 230 mM | 230 mM | 250 mM | 300 mM |

| TIF-IC | TIF-IA | Pol I | TIF-IB | UBF |

B

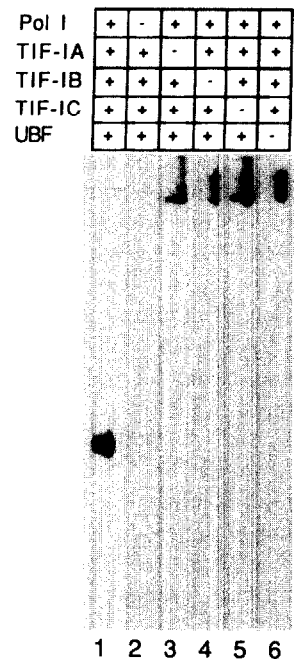|		1	2	3	4	5	6
Pol I		+	−	+	+	+	+
TIF-IA		+	+	−	+	+	+
TIF-IB		+	+	+	−	+	+
TIF-IC		+	+	+	+	−	+
UBF		+	+	+	+	+	−

1 2 3 4 5 6

Fig. 1. Separation and functional identification of multiple factors required for faithful rDNA transcription initiation. (A) Schematic representation of the chromatographic separation of murine pol I transcription factors. The samples were loaded on the individual columns and eluted either stepwise or with gradients. The numbers represent the concentration of KCl (mM). For details, see Experimental. (B) Factors required for transcription from the mouse rDNA promoter. The complete reconstituted transcription assay contained 60 ng of plasmid pMr600 truncated with EcoRI, 5 μl of partially purified pol I, 3 μl of TIF-IA, 2 μl of TIF-IB, 3 μl of TIF-IC and 2 μl of UBF (lane 1). In lanes 2–6 one of the individual fractions (as indicated above the lanes) was omitted from the reactions. The band represents the specific 297 nt run-off transcript.

Ion-exchange chromatography on S-Sepharose

As a first step in the purification protocol, extract proteins were applied to a DEAE-Sepharose column and step eluted at 280 mM KCl. This fraction, which contains pol I and the four transcription factors, was loaded directly on a heparin-Ultrogel column and eluted with 200, 400, 600 and 1000 mM KCl, yielding fractions H-200, H-400, H-600 and H-1000, respectively. Chromatography on the heparin column turned out to be the most essential part of the purification scheme, because it separates pol I and the individual transcription initiation factors. In addition, this step results in a 15-fold purification of pol I with a 10-fold increase in specific activity.

An efficient purification of pol I-containing H-400 fraction was achieved on an S-Sepharose High-Performance column, as shown in Fig. 2. On this column 80% of the protein was removed from the H-400 eluate, resulting in a 3.5-fold increase in specific activity. Hence S-Sepharose HP is a good alternative to Mono-S columns in large-scale preparations, because the resolution on S-Sepharose was nearly as good as that on a Mono S FPLC column.

Gel filtration on HiLoad 26/60

Eukaryotic pol I has a molecular weight of *ca.* 500 000 dalton [19,20]. Because of the large size of pol I, gel filtration or glycerol gradients should be included in the purification procedure. A disadvantage of glycerol gradients is the small amount (about 200–500 μl) of sample which can be applied in the gradient. To overcome this problem a preparative gel filtration column (HiLoad 26/60) was used, which allows the applicaton of up to 13 ml of sample. Prior to application of pol I, the column was calibrated under identical conditions using marker proteins as indicated in Fig. 3.

A HiLoad 26/60 column was equilibrated with buffer AM-100 and 10 ml (3 mg of protein) of active S-Sepharose fractions were applied. As shown in Fig. 3, pol I activity elutes at 132 ml, which corresponds to a molecular weight of about 500 000 dalton. Gel filtration resulted in an 8-fold purification of the enzyme. However, a large decrease in pol I activity (about 50%) was observed, which is probably due to dilution of the enzyme, which results in partial denaturation and inactivation of enzyme activity (after 2 days of storage at $-80°$C the pol I activity was completely lost). Nevertheless, this step
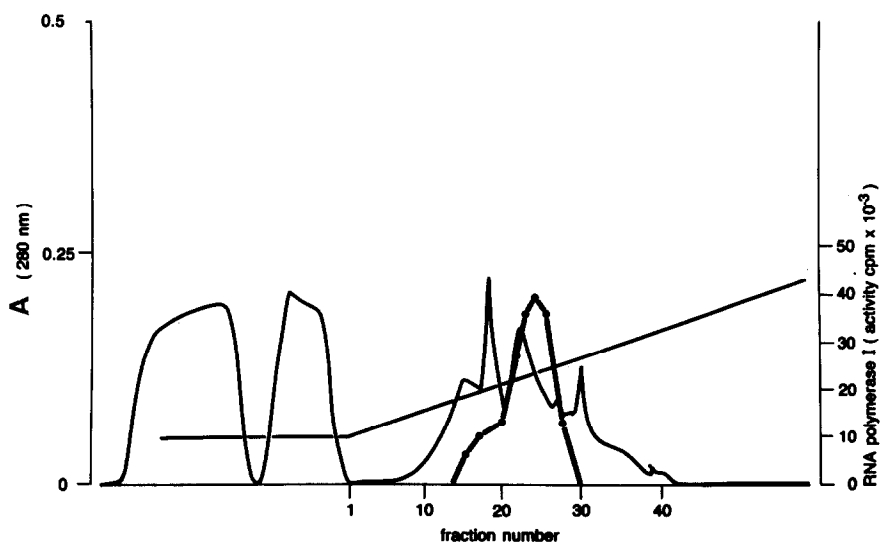

Fig. 2. Purification of RNA polymerase I on S-Sepharose HP. A 90-ml heparin 400 fraction (75 mg of protein) was applied to S-Sepharose High Performance 16/100 (20 ml), equilibrated with three bed volumes of buffer AM 100. Pol I activity was eluted with a gradient from 100 to 450 mM KCl in 250 ml of buffer AM. The flow-rate was 2 ml/min. Fractions of 4 ml were collected and dialysed against buffer AM 100.
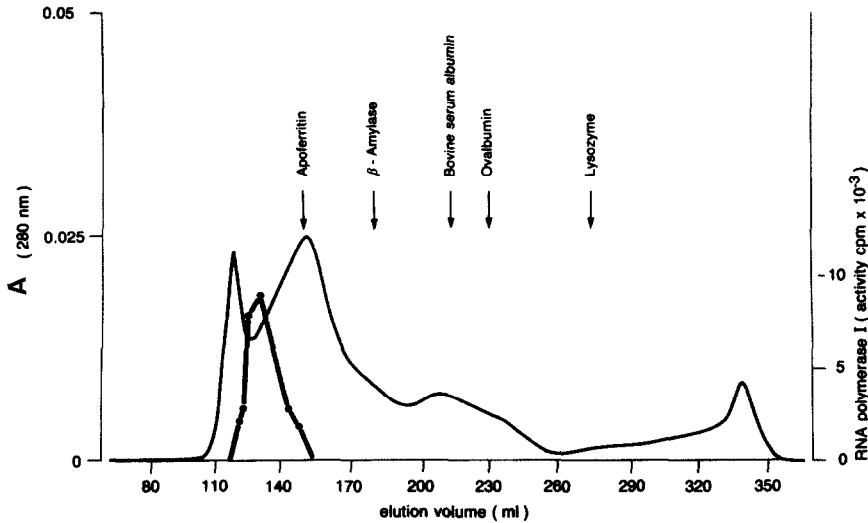
Fig. 3. Gel filtration chromatography of pol I using a HiLoad 26/60 (Superdex 200) column. Fractions of 10 ml of active S-Sepharose were applied to the column, pre-equilibrated with buffer AM-100. The flow-rate was 2 ml/min, pressure 0.3 MPa and fraction size 3 ml. Pol I activity appears at an elution volume of 132 ml. The following standard proteins were used to calibrate the column: apoferritin (443 000 dalton), β-amylase (200 000 dalton), bovine serum albumin (66 000 dalton), ovalbumin (43 000 dalton) lysozyme (14 000 dalton).

turned out to be very important in our purification scheme, because cross-contamination of the DNA binding factors UBF and TIF-IB could be avoided. To stabilize the enzymatic activity, pol I-containing fractions were concentrated on a Mono Q column.

Ion-exchange chromatography on Mono Q

The pool of pol I-containing fractions from the HiLoad 26/60 column was loaded immediately on a Mono Q column, which was equilibrated with buffer AM-100, and the bound proteins were eluted
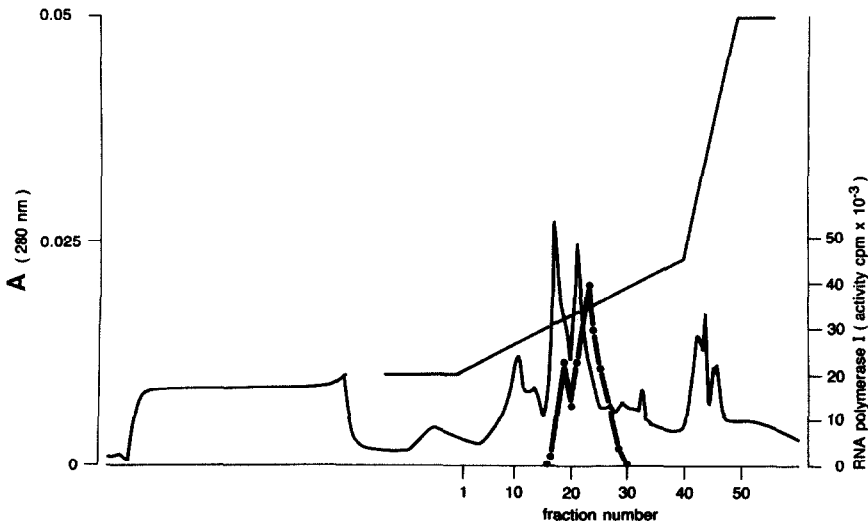

Fig. 4. Anion-exchange chromatography of RNA polymerase I on a Mono Q HR 5/5 column. About 30 ml of pol I-containing fractions size-fractionated on HiLoad 26/60 were loaded on a Mono Q HR 5/5 column. Pol I activity was eluted with a salt gradient from 150 to 450 mM KCl in 20 ml of buffer AM. The flow-rate was 0.5 ml/min, pressure 2 MPa and fraction size 500 μl.

TABLE I

PURIFICATION DATA FOR RNA POLYMERASE I

The data indicate the recovery of RNA polymerase I from 300-ml cell extracts. The purification procedure and the preparation of the cell extracts are described under Experimental.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
Extract	300	1800	55 000	100	30	1
DEAE	150	450	44 000	79	98	3.1
Heparin	30	25	27 000	49	1092	35
S-Sepharose	10	3	11 000	19	3666	120
HiLoad	30	0.7	5500	10	7800	252
Mono Q	2.5	0.18	2300	4	12 670	410

with a gradient from 150 to 450 mM KCl in 20 ml of buffer. A typical elution profile on Mono Q is shown in Fig. 4. Purification of pol I on Mono Q yielded a 4 fold purification and a 2-fold increase in specific activity. Chromatography of pol I on Mono Q is a very effective step to concentrate and therefore to stabilize pol I. After this step, pol I-contain-

ing fractions could be stored for several weeks at − 80°C without a severe loss of activity.

CONCLUSION

The state of purification of pol I in each step is summarized in Table I. Starting from about 300 ml
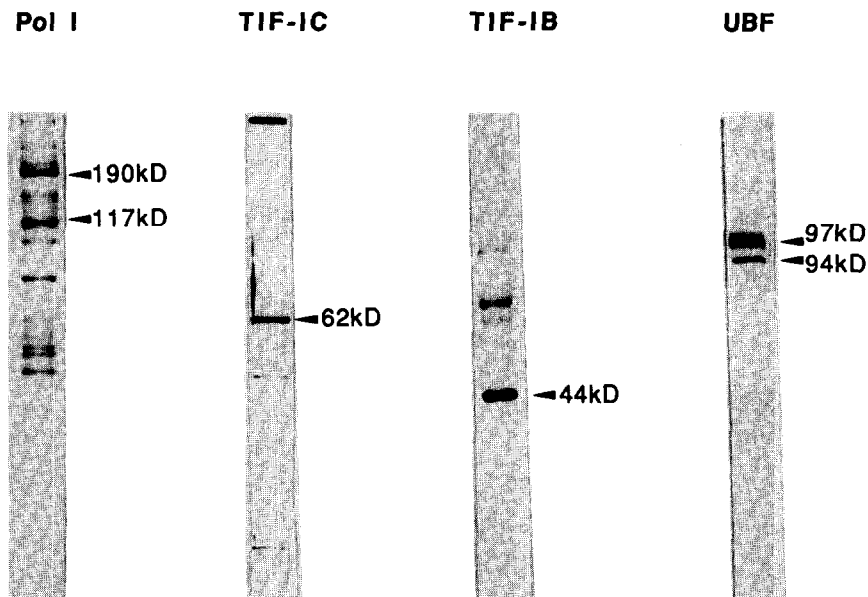

Fig. 5. SDS-PAGE of factors purified according to Fig. 1A. RNA polymerase I: 20 μl of a Mono Q peak fraction were loaded on a 7.5–15% discontinous SDS polyacrylamide gel. The sizes of the two largest subunits of pol I are indicated. TIF-IC: 25 μl of an Orange A fraction were electrophoresed on an 8% SDS polyacrylamide gel. TIF-B: 25 μl of a calf thymus DNA cellulose fraction were electrophoresed on an 11% SDS polyacrylamide gel. UBF: 40 μl of the DNA affinity column fraction were loaded on a 7% SDS polyacrylamide gel. All SDS polyacrylamide gels were silver stained and the molecular weights of the proteins are indicated in kilodalton (kD).

of cell extract (1.8 g of protein), which contained a total pol I activity of 55 000 units, 4% of the total enzyme activity was recovered. The degree of purification obtained was more than 400-fold. This level of purification, however, is not sufficient to obtain a homogeneous pol I preparation. As shown in Fig. 5, a number of discrete protein bands are visible on a silver-stained SDS gel. Nevertheless, the two largest subunits of pol I are clearly visible and their sizes (190 000 and 117 000 dalton) are in agreement with published data [19,20]. In addition, the second largest subunit was identified by the Western blot technique using antibodies directed against the second largest subunit of *Drosophila melanogaster* pol I (data not shown). Fig. 5 also shows the proteins present in the most highly purified UBF, TIF-IB and TIF-IC fractions which were prepared according to the fractionation scheme shown in Fig. 1. UBF, which has been reported to consist of two 97 000- and 94 000-dalton polypeptides [14,15], has been purified to apparent homogeneity. The TIF-IB activity correlates with a 44 000-dalton polypeptide and TIF-IC activity appears to reside within a 62 000-dalton protein. The most purified TIF-IA preparations still show a complex protein pattern (data not shown) and therefore it is not yet possible to attribute TIF-IA activity to a defined polypeptide.

Using the proposed purification scheme, we are now able to use a highly purified *in vitro* system, which makes it feasible to study the transcription initiation process at the rDNA promoter in a detailed manner and to clone individual factors and to determine their precise role in cellular physiology.

ACKNOWLEDGEMENTS

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