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# Function of the Growth-Regulated Transcription Initiation Factor TIF-IA in Initiation Complex Formation at the Murine Ribosomal Gene Promoter

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**Alterations in the rate of cell proliferation are accompanied by changes in the transcription of rRNA genes. In mammals, this growth-dependent regulation of transcription of genes coding for rRNA (rDNA) is due to reduction of the amount or activity of an essential transcription factor, called TIF-IA. Extracts prepared from quiescent cells lack this factor activity and, therefore, are transcriptionally inactive. We have purified TIF-IA from exponentially growing cells and have shown that it is a polypeptide with a molecular mass of 75 kDa which exists as a monomer in solution. Using a reconstituted transcription system consisting of purified transcription factors, we demonstrate that TIF-IA is a bona fide transcription initiation factor which interacts with RNA polymerase I. Preinitiation complexes can be assembled in the absence of TIF-IA, but formation of the first phosphodiester bonds of nascent rRNA is precluded. After initiation, TIF-IA is liberated from the initiation complex and facilitates transcription from templates bearing preinitiation complexes which lack TIF-IA. Despite the pronounced species specificity of class I gene transcription, this growth-dependent factor has been identified not only in mouse but also in human cells. Murine TIF-IA complements extracts from both growth-inhibited mouse and human cells. The analogous human activity appears to be similar or identical to that of TIF-IA. Therefore, despite the fact that the RNA polymerase transcription system has evolved sufficiently rapidly that an rDNA promoter from one species will not function in another species, the basic mechanisms that adapt ribosome synthesis to cell proliferation have been conserved.**

Changes in cell proliferation are accompanied by drastic alterations in the rate of rRNA synthesis. Transcription of the genes encoding rRNA (rDNA) is down-regulated when cells approach stationary phase or are starved of an essential nutrient and is up-regulated upon reversal of such conditions (18). Furthermore, various treatments, such as hormone administration (6, 7, 19, 38) or drug-induced inhibition of protein synthesis (15, 29, 41), alter rDNA transcription. In all of these instances, the rate of in vivo rDNA transcription is reflected in the transcriptional activity of extracts prepared from these cells. The availability of cell-free transcription systems that faithfully mirror the cellular rDNA transcriptional activity facilitates the investigation of the molecular mechanisms underlying this growth-dependent transcriptional control.

Similarly to genes transcribed by RNA polymerase II (Pol II) and Pol III, transcription initiation from the rRNA gene promoter by Pol I is a multistage process requiring the action of at least four transcription factors which assemble at the promoter together with Pol I in an ordered fashion to form active preinitiation complexes. Most of these factors have been extensively purified, and the exact order of assembly into a functional preinitiation complex has been determined elsewhere (33). Two of these factors, TIF-IB and UBF, are DNA-binding proteins which function early in the initiation pathway. Promoter selectivity is conferred by TIF-IB (9, 13), variously termed TFID (39, 40), SL1 (3, 4), or Rib1 (28). TIF-IB is a multisubunit factor containing the TATA box-binding protein (TBP) and three specific TBP-associated factors (10, 13). Transcription is augmented by UBF, the factor which stabilizes binding of TIF-IB to the rDNA promoter. In addition, UBF has been shown to enhance ribosomal gene transcription by relieving repression exerted by a negative-acting factor which competes for binding of TIF-IB to the mouse rDNA promoter (22, 23). Once TIF-IB is tethered to the promoter, Pol I together with the Pol I-associated factors TIF-IC and TIF-IA can bind and form a productive initiation complex (33).

In principle, regulation of rDNA transcription could occur either during the formation of the stable preinitiation complex (presumably involving TIF-IB and UBF) or, alternatively, at a later step prior to initiation. Work from our and other laboratories has led to a consensus that most of the growth-dependent fluctuations of transcriptional activity are mediated by a factor which tightly associates with Pol I and which is required for transcription initiation. This factor, named TIF-IA by our group (5, 34), is probably the same as TFIC, a factor that is responsible for the hormonal regulation of rRNA synthesis in P1798 lymphosarcoma cells (7, 16, 25–27). Both TIF-IA and TFIC show no Pol I activity on their own and, therefore, are true transcription factors for Pol I. The available data suggest that the function of TIF-IA and/or TFIC is inherent in its tight association with Pol I. In this communication, we describe the purification of this regulatory factor, analyze its physicochemical properties, and describe its role in rDNA transcription initiation. We show that binding of TIF-IA to Pol I is a prerequisite for the formation of an initiation-competent enzyme. The association of TIF-IA with the ternary complex consisting of TIF-IB and Pol I plus TIF-IC converts the stable preinitiation complex into a productive initiation complex which in the presence of nucleotides will faithfully synthesize rRNA.

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## MATERIALS AND METHODS

**Cell culture and extract preparation.** Ehrlich ascites cells were cultured in RPMI medium supplemented with 5% newborn calf serum and harvested at the exponential phase of growth. HeLa cells were cultured in Joklik medium supplemented with 5% newborn calf serum. Ehrlich ascites cells were treated for 14 to 16 h with nocodazole (Sigma; 40 ng/ml) prior to extract preparation. Transcriptionally inactive extracts were prepared from stationary-phase cells which were grown to maximal density (about $2 \times 10^6$ cells per ml) and incubated for an additional 24 h before harvesting and extract preparation. Cytoplasmic (S-100) and nuclear extracts were prepared as described elsewhere (33).

**rDNA templates.** The pUC9-based murine rDNA templates pMrSP and pMrWT contain sequences extending from nucleotides −168 to +292 and from −168 to +155, respectively. pMrSP was cleaved with *Eco*RI and pMrWT was cleaved with *Nde*I to generate 297- and 375-nucleotide runoff transcripts, respectively. The template pHrP contains human rDNA sequences from positions −411 to +379 cloned into pUC9. After cleavage with *Eco*RI, 389-nucleotide runoff transcripts are synthesized.

**In vitro transcription assays.** Transcription reactions and product analysis were performed as described elsewhere (33). Assay mixtures (25 μl each) contained 40 ng of template DNA and a total of 15 μl of either cell extracts and/or purified transcription factors. The transcripts were analyzed by gel electrophoresis and quantitated by using a PhosphorImager (Molecular Dynamics). To measure transcription in permeabilized cells, 1 ml of cultured cells ($9 \times 10^5$ cells) was spun down, washed with phosphate-buffered saline, and resuspended in 100 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 75 mM $(NH_4)_2SO_4$, 0.2 M sucrose, 1 mM 1,4-dithioerythritol, and 0.5% saponin. A 5-μl volume of the cell suspension was incubated for 15 min at 37°C in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.9); 5 mM $MgCl_2$; 50 mM $(NH_4)_2SO_4$; 5 mM DTE; 0.6 mM each ATP, GTP, and CTP; 0.025 mM [$^3$H]UTP; and 200 μg of α-amanitin per ml. Reactions were stopped by the addition of 0.2 ml of saturated $Na_4P_2O_7$ and were precipitated with 5% trichloroacetic acid. The precipitates were collected on glass fiber filters and quantified by scintillation counting.

**Purification of TIF-IA.** TIF-IA was purified from 1.6 liters of a mixture of nuclear and cytoplasmatic extracts obtained from about $4 \times 10^{11}$ cultured Ehrlich ascites cells. The buffers used were either buffer A (20 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 20% glycerol, 0.5 mM DTE, and 0.5 mM phenylmethylsulfonyl fluoride) or buffer AM (same as buffer A, but with 5 mM $MgCl_2$) containing different concentrations of KCl. First, 100 ml of extract was fractionated on an 80-ml DEAE-Sepharose CL-6B column by step elution with 280 mM KCl in buffer A. After dilution to 200 mM KCl, eluates from three individual columns were pooled and fractionated on an 80-ml heparin-Ultrogel A4R column. Bound proteins were eluted by steps of 0.25, 0.4, 0.6, and 1 M KCl in buffer AM, yielding fractions H-200, H-250, H-400, H-600, and H-1000, respectively. TIF-IA and TIF-IC present in the H-200 and H-250 fractions were concentrated by binding to and elution with 300 mM KCl from Q-Sepharose (80 ml). The eluate (QS-300) was applied to a Mono Q (HR 16/10) column (Mono Q1), and bound proteins were recovered with 60 ml of a linear gradient ranging from 200 to 300 mM KCl. TIF-IA, which eluted at 230 mM KCl, was dialyzed against buffer AM-75 and fractionated on ω-amino-octyl-agarose (30 ml). TIF-IA eluted at 150 mM KCl from this matrix. TIF-IA containing fractions from five different preparations were pooled and precipitated by the addition of solid $(NH_4)_2SO_4$ (0.194 g/ml). The precipitate was collected by centrifugation at 12,000 × *g* for 45 min. After dialysis against buffer AM-100, the 35% ammonium sulfate fraction was chromatographed on a polyethyleneimine high-pressure liquid chromatography (HPLC) column. After being washed with AM-250, TIF-IA was eluted with a 50-ml linear gradient from 250 to 1,000 mM KCl. TIF-IA activity eluted at about 700 mM KCl. The next step in purification involved gel filtration on a preparative Superdex 200 column (HiLoad 26/60). Active fractions were recovered between 190 and 240 ml, which corresponds to a native molecular mass of between 60 and 100 kDa. These fractions were immediately concentrated on a Mono Q (HR 5/5) column (Mono Q2). Bound proteins were eluted with a 12-ml shallow linear salt gradient ranging from 200 to 300 mM KCl.

For partial purification of human TIF-IA, 220 ml of a mixture of nuclear and cytoplasmic extracts derived from cultured HeLa cells was fractionated on DEAE-Sepharose and Heparin-Ultrogel as described for the mouse factors, except that both columns were run in buffer AM. By this purification strategy, TIF-IA activity present in the H-400 fraction was further purified by chromatography on a Mono Q column. On this column, the peak of TIF-IA activity eluted at 250 mM KCl.

**Estimation of the native molecular mass of TIF-IA by gel filtration.** The native molecular mass of TIF-IA was determined by size exclusion chromatography either on an analytical Superdex-200 fast-performance liquid chromatography (FPLC) column or on a GF-250 HPLC column (Du Pont). A 200-μl volume of TIF-IA (Mono Q2 fraction) was applied to either column. TIF-IA activity eluted at about 8.3 ml from the GF-250 matrix and at about 13.1 ml from the Superdex-200 matrix, corresponding to a native molecular mass of 70 to 80 kDa. The molecular mass standards used for calibration were galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

**Measurement of protein kinase activity.** Extract proteins (150 μg) obtained from either untreated or nocodazole-arrested cells were mixed with 2 μl of the anti-Cdc2 antiserum G6 (12) in a total volume of 100 μl in RIPA buffer (10 mM Tris-HCl [pH 7.2], 100 mM KCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate). After incubation for 1.5 h at 4°C, 10 μl of protein A-Sepharose was added, and incubation was continued for an additional 30 min. The collected immunocomplexes were washed three times with 500 μl of RIPA buffer and once with 500 μl of reaction buffer (10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 50 mM KCl, 100 μM $Na_3VO_4$, 0.5 mM NaF) and were assayed for protein kinase activity in 20 μl of reaction buffer in the presence of either histone H1 (30 μg) or casein (30 μg) and 10 μM [γ-$^{32}$P]ATP (20,000 dpm/pmol). After incubation for 15 min at 30°C, reactions were stopped and phosphorylated proteins were analyzed by autoradiography.

## RESULTS

**TIF-IA activity is lacking in extracts from stationary cells.** The rDNA transcriptional activities of extracts prepared from exponentially growing cells and of extracts prepared from stationary-phase cells are shown in Fig. 1A. Clearly, specific transcription in extracts from growth-arrested cells is reduced relative to that of extracts from exponentially growing cells (lanes 1 and 2). Both transcriptionally active

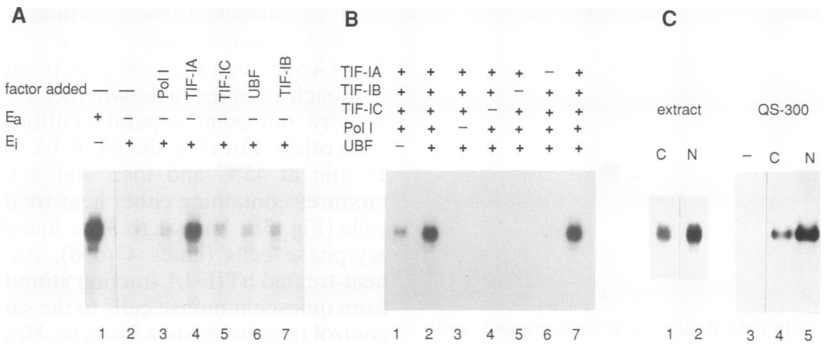



FIG. 1. TIF-IA is an essential rDNA transcription factor. (A) Complementation of transcriptional activity in extracts from stationary-phase cells by TIF-IA. The assay mixtures contained 5 μl of extracts prepared either from growing cells ($E_a$ [lane 1]) or from stationary-phase cells ($E_i$ [lanes 2 to 7]). To reconstitute transcriptional activity, the inactive extract was supplemented with 9 μl of Pol I or with 9 μl of the individual factors indicated above the lanes. (B) Transcription in the reconstituted transcription system. The reaction mixtures contained 20 ng of template pMrWT/*Nde*I and the individual protein fractions listed above the lanes. (C) Extracts from mitotic cells contain high levels of TIF-IA. Lanes 1 and 2, transcriptional activities of extracts derived from cells that were treated with dimethyl sulfoxide (C) or nocodazole (N); lanes 3 to 5, Extracts from dimethyl sulfoxide (C)- or nocodazole (N)-treated cells were fractionated in parallel on DEAE-Sepharose, Heparin-Ultrogel, and Q-Sepharose. The Q-Sepharose peak fractions were assayed for TIF-IA activity by supplementing 5 μl of extract from stationary-phase cells (lane 3) with 8 μl of the corresponding fraction (lanes 4 and 5).

and inactive extracts contain the same amount of potentially active Pol I, as judged by α-amanitin-resistant transcription on denatured calf thymus DNA (data not shown). The transcriptional activity is restored by supplementing extracts from growth-arrested cells with a partially purified TIF-IA fraction (lane 4). Addition of Pol I, TIF-IB, TIF-IC, or UBF, on the other hand, had no effect (lanes 3 and 5 to 7).

The availability of purified transcription factors enabled us to assay the functional role of TIF-IA in a reconstituted transcription system. Each of the individual protein fractions used has been purified on at least four different chromatographic columns. Transcription in this reconstituted system was strictly dependent on the presence of Pol I, TIF-IA, TIF-IB, and TIF-IC, indicating that these proteins serve an indispensable function in rDNA transcription (Fig. 1B, lanes 2 to 7). Omission of UBF, on the other hand, caused only a fivefold reduction of transcriptional activity (lane 1). This relatively modest effect of UBF in the presence of highly purified transcription factors supports our previous finding that the strong UBF-mediated transcriptional activation observed in a partially purified reconstituted system (50- to 100-fold) is caused by an antirepression mechanism (22, 23). Importantly, TIF-IA activity could be replaced neither by Pol I nor by any other factor and, therefore, is clearly distinct from any other component in our transcription system.

**Extracts from cells arrested in mitosis contain high levels of TIF-IA.** Since the transcriptional activities of extracts derived from growing and resting cells mirror regulation of rDNA transcription that occurs in vivo, we wondered whether this correlation between cellular rRNA synthesis and TIF-IA levels is also observed in extracts from mitotic cells in which transcription is shut off (42). Cells were treated for 15 to 18 h with nocodazole, a drug which arrests cells at an early phase of mitosis. This treatment resulted in approximately 80 to 90% of mitotic cells, as judged by microscopy and by measurement of the activity of the MPF-kinase (data not shown). As expected, cellular rDNA transcription (as assayed in permeabilized cells in the presence of high concentrations of α-amanitin) was decreased in nocodazole-treated cells to 15 to 30% compared with untreated cells (data not shown). Surprisingly, extracts prepared from nocodazole-treated cells exhibited a transcriptional activity that was two- to threefold higher than that of control extracts (Fig. 1C, lanes 1 and 2). To find out whether this increased transcriptional activity in extracts from mitotic cells was due to elevated levels of TIF-IA, this activity was partially purified from control and nocodazole-treated cells. In this and the following experiments, TIF-IA activity was monitored by its ability to stimulate transcription of inactive extracts prepared from stationary cells (Fig. 1C, lanes 3 to 5). Similarly to the increased transcriptional activity of nocodazole-treated extracts, the amount or activity of TIF-IA (QS-300 fractions) recovered from the mitotic cells was about threefold higher than the TIF-IA activity from control cells. This finding indicates that the shutoff of cellular rDNA transcription during mitosis does not involve a reduction of the amount or activity of TIF-IA.

**TIF-IA is not species specific.** One of the most remarkable features of ribosomal gene transcription is its pronounced species specificity. Extracts from mouse cells fail to transcribe human rDNA. Human cell extracts, on the other hand, are not able to transcribe mouse rDNA templates, indicating that one or more proteins required for Pol I transcription initiation are specific for a given organism (3, 4, 17). Therefore, we investigated whether TIF-IA is restricted to mouse cells or whether a similar activity is present also in human cells.

First, the effect of TIF-IA was tested in a heterologous system containing human rDNA and extracts prepared from slowly growing HeLa cells (Fig. 2, lanes 1 and 2). In several extracts tested, a significant stimulation of human rDNA transcription was observed in the presence of mouse TIF-IA (mTIF-IA), indicating that TIF-IA functions also in human Pol I transcription. To perform the reciprocal experiment, i.e., stimulation of mouse cell extracts by human TIF-IA (hTIF-IA), an analogous activity was partially purified from logarithmically growing HeLa cells by using a fractionation scheme similar to that of mTIF-IA. As shown in Fig. 2, lanes 3 and 4, supplementing stationary-phase mouse cell extracts with fractions containing hTIF-IA rescued transcriptional activity. Therefore, this growth-dependent regulatory activity appears to be present in both human and mouse cells and can be exchanged between the two species.

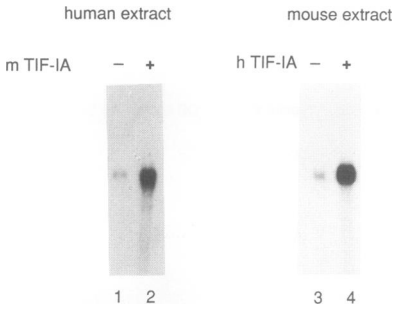



FIG. 2. Transcriptional stimulation of mouse and human cell extracts by TIF-IA. Lanes 1 and 2, stimulation of inactive human extract by mTIF-IA. Extract prepared from slowly growing HeLa cells was assayed for transcriptional activity either in the absence (lane 1) or in the presence (lane 2) of partially purified mTIF-IA (polyethyleneimine fraction). The human rDNA template was pHrP/*Eco*RI (70 ng). Lanes 3 and 4, stimulation of mouse extract by hTIF-IA. HeLa cell extracts were fractionated on DEAE-Sepharose, Heparin-Ultrogel, and Mono Q as described in Materials and Methods. A 9-μl volume of a Mono Q fraction was assayed for TIF-IA activity by complementing 6 μl of inactive mouse extract in the presence of the mouse rDNA template pMrWT/*Nde*I (40 ng).

Similarly to its murine counterpart, partially purified hTIF-IA was found in fractions which contained part of cellular Pol I activity. To prove that hTIF-IA is a true transcription factor and not an activated subform of Pol I, we selectively inactivated Pol I by mild heat treatment. Previous studies have shown that upon heating of extracts to 45°C, Pol I activity decays with a half-life of about 1 min, whereas the half-life of TIF-IA is considerably longer (5, 26). This observation is documented in Fig. 3A, which shows loss of transcriptional activity by heat treatment of active cell extracts (lanes 1 and 2). Addition of Pol I (lane 6), but none of the other factors (lanes 7 to 10), restored the activity of heat-inactivated extract. Moreover, addition of heated extract to extracts from growth-arrested cells reconstituted transcriptional activity (lanes 3 and 4), indicating that TIF-IA survived the mild heat treatment. We have used this approach to investigate whether Pol I and the hTIF-IA-like activity represent separate entities that are distinct from each other. For this, the hTIF-IA fraction was incubated for 15 min at 45°C and then added to transcription reaction mixtures containing either heat-treated extract from growing cells (Fig. 3B, lanes 1 to 3) or inactive extract from stationary-phase cells (lanes 4 to 6). As shown in Fig. 3B, the heat-treated hTIF-IA fraction stimulated the inactive extract from quiescent mouse cells to the same level as the untreated control (compare lanes 5 and 6). However, the same fraction did not stimulate transcription of a heated extract (lane 3), a finding which suggests that hTIF-IA is not an activated subpopulation of Pol I but a distinct factor.

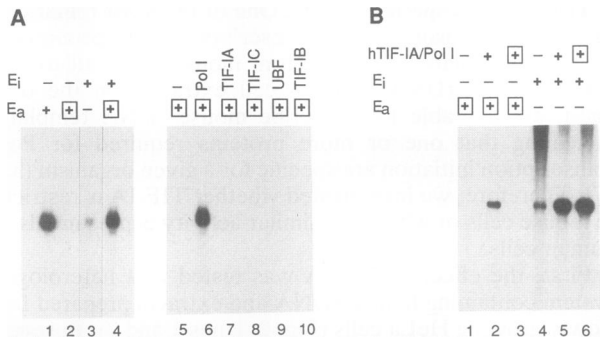
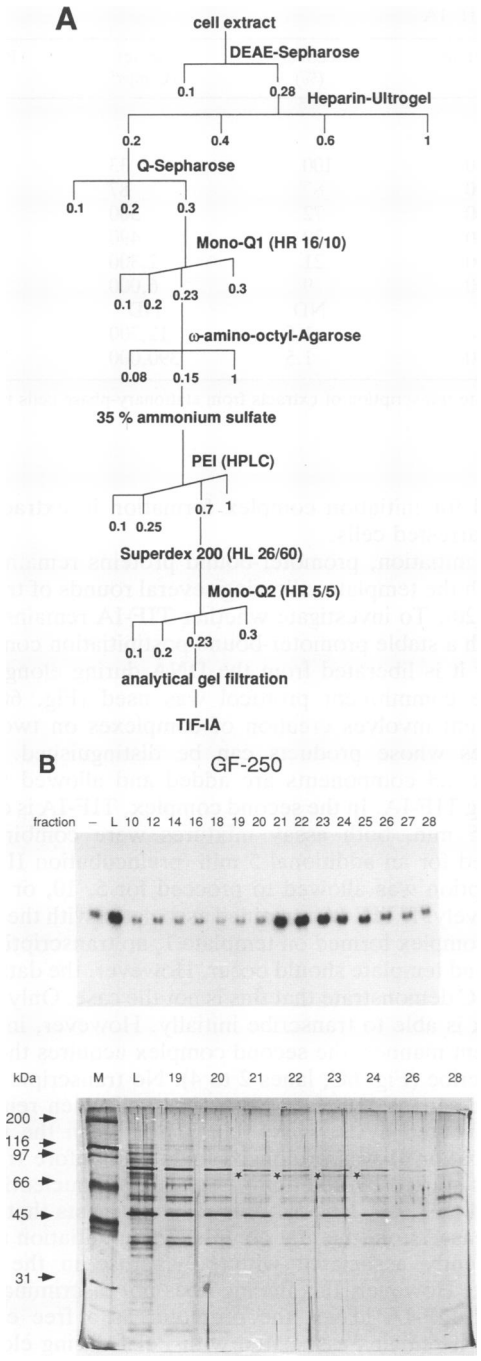



FIG. 3. TIF-IA is more heat resistant than Pol I. (A) Heat treatment of cell extracts. Lanes: 1 and 2, activities of an extract (4 μl) prepared from growing mouse Ehrlich ascites cells ($E_a$) before and after incubation for 15 min at 45°C; 3, activity of extract (6 μl) derived from stationary-phase mouse cells ($E_i$); 4, mixture of 6 μl of extract $E_i$ with 4 μl of heat-treated extract $E_a$; 5 to 10, 5 μl of the indicated protein fractions added to 4 μl of heat-treated extract $E_a$ (marked by squares). (B) Heat treatment of hTIF-IA. A 6-μl volume of a Mono Q fraction containing both hTIF-IA and small amounts of Pol I was added to 4 μl of heat-treated mouse extract $E_a$ (lanes 1 to 3) or to 6 μl of extract derived from growth-arrested mouse cells ($E_i$) (lanes 4 to 6), either before (lanes 2 and 5) or after (lanes 3 and 6) heat treatment for 15 min at 45°C. The square marks the samples that were heat treated.

**Purification of TIF-IA.** The elucidation of the functional role of TIF-IA in rDNA transcription initiation requires purification and functional characterization of this protein. We have isolated TIF-IA activity from logarithmically growing cells which were treated with nocodazole to increase TIF-IA activity (Fig. 1C). Purification of TIF-IA proved to be very difficult. First, TIF-IA bound only to positively charged matrices. Second, even in rapidly growing cells, the amount of TIF-IA is extremely low. Thus, a typical preparation of TIF-IA started from 1.6 liters of extracts (15 g of protein) prepared from about $4 \times 10^{11}$ cells. The purification scheme is displayed in Fig. 4A. After purification by nine chromatographic steps, at least a 12,000-fold increase in the specific activity of TIF-IA was achieved (Table 1). The total recovery of activity, however, was very low (about 1.5%).

The last step in the purification procedure involved size exclusion chromatography either on a GF-250 HPLC column or on a Superdex-200 FPLC column. On both columns, TIF-IA activity was recovered in fractions corresponding to an apparent molecular mass of approximately 70 to 80 kDa (Fig. 4B and C). To identify the polypeptide(s) which represents TIF-IA, the protein compositions of active fractions of two individual preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 4B and C [lower panels]). The most purified active fractions still contain several polypeptides. None of these proteins, except a 75-kDa polypeptide, correlates with TIF-IA activity. The patterns of the contaminating proteins varied considerably among individual preparations. The 75-kDa polypeptide, however, was detected in all highly purified TIF-IA preparations and exactly correlated with TIF-IA activity. Despite the high input of starting material and extensive purification, this 75-kDa protein represents only a minor part of the proteins which are present in the peak fractions. The size of the 75-kDa polypeptide seen on denaturing polyacrylamide gels corresponds to the native molecular mass of 70 to 80 kDa as estimated by gel filtration. This result suggests that TIF-IA is a globular monomeric factor consisting of one 75-kDa polypeptide.

**TIF-IA interacts with Pol I in solution.** Previously, we have shown that binding of TIF-IA to preinitiation complexes is the last step in the formation of productive initiation complexes (33). This final assembly step appears to occur via specific interactions between TIF-IA and Pol I. However, these experiments did not unambiguously prove whether TIF-IA bound directly to Pol I or whether this protein-protein interaction was mediated by the Pol I-associated factor TIF-IC. To address this point, the elution of either TIF-IA alone or after incubation with Pol I was analyzed on a GF-250 sizing column. Once again, TIF-IA activity eluted



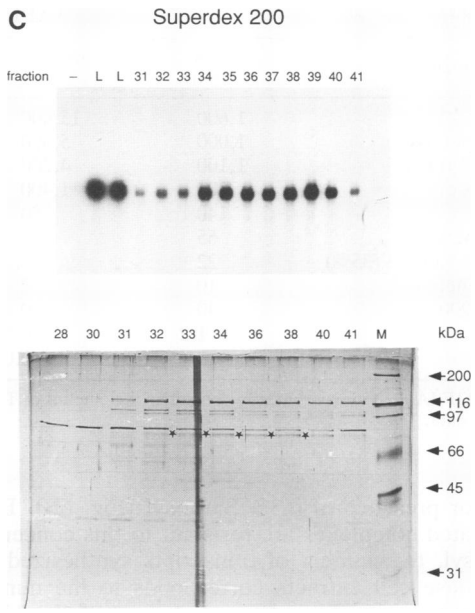




FIG. 4. Purification of TIF-IA. (A) Schematic representation of the protocol used for the purification of TIF-IA. (B) Size exclusion chromatography of TIF-IA on GF-250. (Upper panel) TIF-IA activity of GF-250 column fractions. A 8-μl volume of the column fractions indicated above the lanes was assayed for TIF-IA activity. Lanes – and L are controls showing the activity of the inactive extract (5 μl) in the absence of TIF-IA (–) or in the presence of 4 μl of the fraction applied to the column (L). (Lower panel) SDS-PAGE analysis of proteins present in GF-250 fractions. A 40-μl volume of the indicated fractions was electrophoresed on an SDS–10% polyacrylamide gel, and proteins were visualized by silver staining. Molecular weight markers are indicated by arrows. The 75-kDa polypeptide copurifying with TIF-IA is marked by asterisks. β-Galactosidase (116 kDa), phosphorylase (97 kDa), and bovine serum albumin (66 kDa) elute in fractions no. 16, no. 19, and no. 25, respectively. (C) Size exclusion chromatography of TIF-IA on Superdex-200. (Upper panel) TIF-IA activity of Superdex-200 column fractions. Assays were performed as described for panel B. (Lower panel) SDS-PAGE analysis. A 40-μl volume of fractions eluting from the column was electrophoresed on an SDS-polyacrylamide gel, and proteins were visualized by silver staining. Molecular weight markers are indicated by arrows. The 75-kDa polypeptide copurifying with TIF-IA is marked by asterisks.

at a volume corresponding to about 70 to 80 kDa (Fig. 5A). However, in the presence of Pol I, two peaks of TIF-IA activity were detected, one eluting in the void volume and one eluting at about 80 kDa (Fig. 5B). Since the Pol I fraction used exhibits no TIF-IA activity on its own, the presence of TIF-IA activity in the void volume is probably due to formation of a binary complex between Pol I and TIF-IA, which suggests that association with Pol I occurs in the absence of any other factor.

**Function of TIF-IA in transcription initiation.** If TIF-IA is a bona fide initiation factor that acts prior to chain elongation, it should be required for the formation of the first phosphodiester bonds of nascent rRNA. To prove this assumption, purified factors were incubated with template DNA in the presence of ATP and CTP, which are the first two nucleotides of mouse pre-rRNA. Then, [$^{32}$P]UTP (the third nucleotide) was added, and the formation of labelled ACU trimers was analyzed by gel electrophoresis. As shown in Fig. 6A, trimer synthesis was dependent on the presence of template DNA and all five protein factors. Omission of TIF-IA prevented synthesis of the trimer, i.e., the formation of the second internucleotide bond, a finding which underscores the essential role of TIF-IA during early steps of transcription initiation.

Since TIF-IA was the only factor missing in extracts from growth-arrested cells, such extracts should be unable to assemble productive initiation complexes at the rDNA promoter. The formation of initiation complexes can be assayed by their relative resistance against the detergent Sarkosyl (33). Therefore, we analyzed TIF-IA-mediated transcription in extracts from growing or growth-arrested cells in the

TABLE 1. Purification of TIF-IA

| Fraction | Vol (ml) | Amt of protein (mg) | TIF-IA activity (U)[a] | Recovery (%) | Sp act (U/mg)[a] | Purification (fold) |
|---|---|---|---|---|---|---|
| Extract | 1,600 | 15,000 | | | | |
| DEAE-Sepharose | 1,000 | 5,000 | | | | |
| Heparin-Ultrogel | 1,100 | 4,200 | 140,000 | 100 | 33 | |
| Q-Sepharose | 200 | 1,400 | 122,000 | 87 | 87 | 2.6 |
| Mono-Q1 | 140 | 280 | 101,000 | 72 | 360 | 11 |
| ω-Amino-octyl | 65 | 110 | 54,200 | 39 | 490 | 15 |
| Ammonium sulfate (35%) | 22 | 13.2 | 30,000 | 21 | 2,300 | 70 |
| Polyethyleneimine | 10 | 2 | 12,000 | 9 | 6,000 | 180 |
| Superdex-200 | 40 | 0.2 | ND[b] | ND | ND | ND |
| Mono-Q2 | 1.2 | 0.15 | 2,000 | 1.5 | 12,700 | 390 |
| GF-250 | 2.4 | 0.005 | 1,900 | 1.5 | 390,000 | 11,800 |

[a] 1 U of TIF-IA activity is arbitrarily defined as the amount of TIF-IA required to stimulate transcription of extracts from stationary-phase cells twofold.
[b] ND, no data.

absence or presence of 0.3% Sarkosyl (Fig. 6B). Because only initiated complexes are resistant to this concentration of Sarkosyl, the number of transcripts synthesized in stationary-phase cell extracts corresponds to the number of active initiation complexes formed. As expected, addition of exogenous TIF-IA to extracts from growing cells affected neither the overall transcriptional activity nor the number of Sarkosyl-resistant complexes (Fig. 6B, lanes 1 to 4), indicating that in such extracts TIF-IA was not a limiting factor. In stationary-phase cell extracts, on the other hand, stimulation of overall transcription by exogenous TIF-IA (Fig. 6B, lanes 5 and 6) was accompanied by a drastic increase in the number of Sarkosyl-resistant initiation complexes, which was comparable to that of the control extract (compare lanes 4 and 8). Thus, preinitiation complexes can be formed in the absence of TIF-IA, but initiation is precluded. Furthermore, this result indicates that TIF-IA is the only limiting factor required for initiation complex formation in extracts from growth-arrested cells.

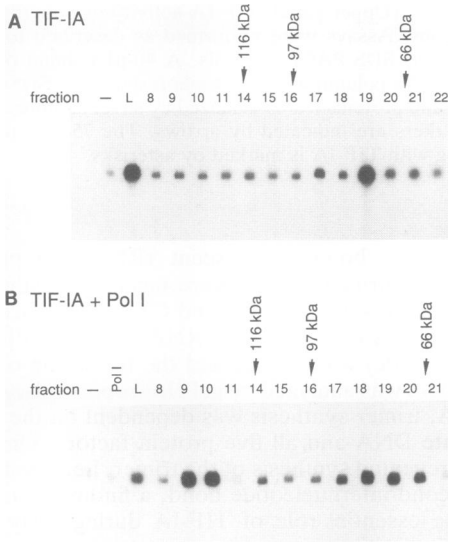



FIG. 5. TIF-IA associates with Pol I in solution. A 120-μl volume of TIF-IA (Mono-Q2 fraction) was either diluted with 80 μl of buffer AM-100 (A) or was incubated with 80 μl of Pol I for 30 min at 30°C (B) prior to chromatography on GF-250. Fractions eluting from both columns were assayed for their ability to stimulate transcription of extract prepared from stationary-phase cells. Each assay mixture contained 6 μl of inactive extract and 9 μl of the fractions indicated above the lanes.

After initiation, promoter-bound proteins remain associated with the template, allowing several rounds of transcription (1, 20). To investigate whether TIF-IA remains associated with a stable promoter-bound postinitiation complex or whether it is liberated from the DNA during elongation, a template commitment protocol was used (Fig. 6C). The experiment involves creation of complexes on two rDNA templates whose products can be distinguished. In one complex, all components are added and allowed to bind, including TIF-IA. In the second complex, TIF-IA is omitted. After 15 min, both assay mixtures were combined and incubated for an additional 5 min (preincubation II) before transcription was allowed to proceed for 5, 10, or 15 min, respectively. If TIF-IA remained associated with the postinitiation complex formed on template 1, no transcription from the second template should occur. However, the data shown in Fig. 6C demonstrate that this is not the case. Only the first complex is able to transcribe initially. However, in a time-dependent manner, the second complex acquires the ability to transcribe (Fig. 6C, lanes 2 to 4). No transcripts derived from the second template were observed when reinitiation was prevented by Sarkosyl (lane 1) or when the time for preincubation II was prolonged to 15 min before transcription was started by addition of the missing nucleotides and Sarkosyl (Fig. 6C, lane 5). This result suggests that TIF-IA was released from the first complex upon initiation and was subsequently associated with polymerase in the second complex. However, this finding does not discriminate as to whether TIF-IA leaves the promoter as a free entity or whether it remains associated with Pol I during elongation and reinitiation.

**TIF-IA increases the rate of reinitiation.** The results presented above underscore the essential role of TIF-IA in initiation complex formation and suggest that this factor is required for promotion of multiple rounds of transcription. To address this point, initiation complexes were formed by incubating template DNA with constant amounts of Pol I, TIF-IB, TIF-IC, and UBF in the presence of increasing amounts of TIF-IA. Transcription was allowed to proceed in either the absence or the presence of 0.045% Sarkosyl. At this detergent concentration, reinitiation is precluded, and the amount of Sarkosyl-resistant transcripts is, therefore, an estimate of the number of initiation complexes formed. Moreover, since each stable promoter complex may promote several rounds of transcription, the number of tran-

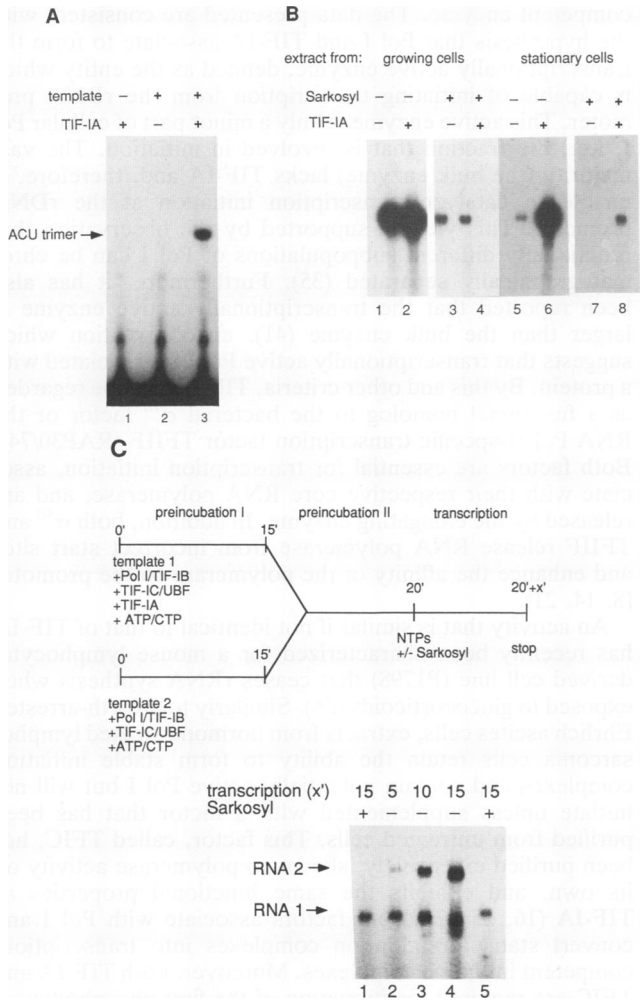



FIG. 6. Functional properties of TIF-IA. (A) TIF-IA is required for the formation of the first phosphodiester bonds. Pol I and transcription factors were incubated for 20 min at 30°C together with the rDNA template and ATP and CTP prior to the addition of [$\alpha$-$^{32}$P]UTP and then further incubated for 25 min. To obtain a better resolution between the labelled mononucleotides and the ACU trimers, the reaction mixtures were treated with 1.5 U of alkaline phosphatase for 15 min before the reaction products were analyzed on a 25% denaturing gel. The formation of ACU trimers was visualized by autoradiography. Lanes 1 and 2 are controls showing that the synthesis of trimers is dependent on the presence of the rDNA template and TIF-IA. (B) Extracts prepared from stationary-phase cells are unable to form initiated complexes. Assay mixtures contained 50 $\mu$g of extract proteins obtained from exponentially growing cells (lanes 1 to 4) or from stationary-phase cells (lanes 5 to 8), 50 ng of template pMrSP/*Eco*RI and, when indicated, 8 $\mu$l of TIF-IA (Mono-Q2 fraction). Prior to transcription, the extracts were preincubated with ATP and CTP either in the absence (odd-numbered lanes) or in the presence (even-numbered lanes) of TIF-IA. After 20 min, transcription was started by the addition of the missing nucleotides, and 0.3% Sarkosyl was added to reaction mixtures 3, 4, 7, and 8. Reactions were stopped after 60 min. (C) TIF-IA is not an integral part of the postinitiation complex. Assays were performed as diagrammed, except that in lane 5, preincubation II was prolonged to 15 min before addition of the missing nucleotides and Sarkosyl. Template 1 was pMrSP/*Eco*RI, and template 2 was pMrWT/*Nde*I. Each reaction mixture contained 25 ng of both rDNA templates, 2 $\mu$l of Pol I, 2 $\mu$l of TIF-IB, 1 $\mu$l of TIF-IC, 0.5 $\mu$l of UBF, 0.75 $\mu$l of TIF-IA (Mono-Q2 fraction), and, when indicated, 0.045% Sarkosyl during the transcription period to preclude reinitiation.

scripts synthesized from a given number of initiation complexes directly reflects the rate of reinitiation. As shown in Fig. 7, at low concentrations of TIF-IA (from 0.5 to 1.5 $\mu$l), approximately the same amounts of transcripts were synthesized in the presence or absence of Sarkosyl. This observation supports the previous data showing that TIF-IA stimulates transcription by increasing the number of initiation complexes. Further increase of TIF-IA (above 1.5 $\mu$l) did not increase the number of Sarkosyl-resistant complexes, indicating that, at these TIF-IA concentrations, another factor(s) required for complex formation is limiting. Interestingly, under these conditions, elevated levels of TIF-IA strongly stimulated overall transcription. This apparent difference in the total number of assembled initiation complexes and the absolute number of transcripts synthesized suggests that excess of TIF-IA stimulates the rate of reinitiation and augments multiple rounds of transcription.

## DISCUSSION

The assembly of functional rDNA transcription initiation complexes is a highly ordered process which involves the stepwise association of several proteins with the ribosomal gene promoter. Pol I is recruited to the promoter by specific protein-protein interactions with the components of the committed complex, i.e., TIF-IB and probably UBF (33). However, this preinitiation complex starts rDNA transcription only after TIF-IC and TIF-IA have bound. Only the complete complex consisting of all four factors and Pol I shows resistance to intermediate concentrations of Sarkosyl (0.045%) and is competent to catalyze the formation of the first phosphodiester bond (33). In principle, transcription initiation could be regulated at each of the individual levels of complex formation, i.e., during promoter recognition, polymerase recruitment, or association of TIF-IA or TIF-IC with the ternary complex. However, the data presented in previous studies and in the present communication demon-

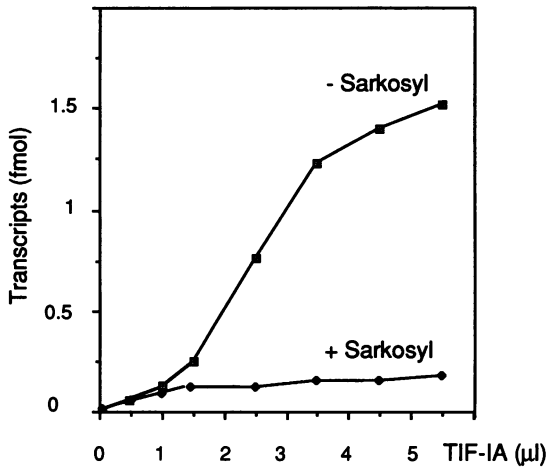



FIG. 7. TIF-IA promotes multiple rounds of transcription. Initiation complexes were assembled in the presence of 80 ng of pMrSP/*Eco*RI, 4 $\mu$l of Pol I, 3 $\mu$l of TIF-IB, 3 $\mu$l of TIF-IC, 1 $\mu$l of UBF, and increasing amounts of TIF-IA (Mono-Q2 fraction) in a total volume of 40 $\mu$l. After 15 min, each assay mixture was divided into two halves. One-half of each reaction mixture was supplemented with nucleoside triphosphates (open squares); the other half was supplemented with nucleoside triphosphates and 0.045% Sarkosyl (closed squares). In both types of reaction, transcription was allowed to proceed for 60 min.

strate that growth-dependent fluctuations in ribosomal gene transcription are mediated by TIF-IA, a factor which binds to Pol I and is required for initiation. In both humans and mice, polymerase will bind but cannot initiate without the subsequent binding of TIF-IA. TIF-IA sets up a productive transcription initiation complex, promotes formation of the first phosphodiester bonds, and plays an important role in reinitiation of transcription. By these criteria, TIF-IA is a bona fide transcription initiation factor. However, it is not known how the amount or activity of TIF-IA is modulated in response to environmental changes.

A different mechanism of rDNA transcriptional regulation appears to be accomplished in the lower eukaryote *Acanthamoeba castellanii*. During starvation or encystment, the rate of rDNA transcription is down-regulated. This activity loss could be reproduced with purified components, and it was shown that it was the polymerase fraction that lost activity when *A. castellanii* entered stationary phase. Pol Is purified from growing cells or cysts differed in their abilities to specifically initiate rDNA transcription (2, 32). In addition, both enzymes had different heat stabilities, a finding which suggested that Pol I itself was modified (32). Recent structural studies have demonstrated changes in the electrophoretic mobility of the 39-kDa subunit that correlate with both down- and up-regulation (31). Therefore, polymerase modification may specifically affect the interaction of Pol I with a transcription factor(s) bound at the promoter. The causal relationship between this finding and those for the mammalian system is not clear. As has been pointed out by Paule (30), one important difference between the modes of regulation is the stage that seems to be blocked by the inactive component. In *A. castellanii*, polymerase will not bind to the promoter when in its inactive state. In growth-arrested mammalian cells, Pol I will bind, but it cannot initiate without the subsequent binding of TIF-IA. Presumably, a mechanism similar to that used by *A. castellanii* may be realized to accomplish transcriptional regulation during cellular differentiation.

Studies of the mechanism whereby TIF-IA participates in Pol I-directed transcription initiation requires the structural and functional characterization of this factor. We have purified TIF-IA using a protocol that employs a combination of several chromatographic steps which are based on ion-exchange and size exclusion columns. The final factor preparation retains the ability to reconstitute transcription both in extracts from stationary cells and in a purified transcription system and is devoid of detectable Pol I activity. In several factor preparations, a 75-kDa polypeptide exactly correlates with TIF-IA activity. Because of the lability and low abundance of this factor, the total yield and recovery of TIF-IA are extremely low. We have obtained approximately 200 ng of the 75-kDa polypeptide from about $4 \times 10^{11}$ cells. When a final yield of 1.5% is taken into account, this amount corresponds to approximately 500 to 1,000 molecules of TIF-IA in the cell. This value is much lower than the number of Pol I molecules per cell (10,000 to 40,000) but compares with the values estimated for other Pol I-specific transcription factors such as SL1/TIF-IB (13, 24) or the murine rDNA termination factor TTFI (36). The low stoichiometry of TIF-IA compared with that of Pol I supports our view that TIF-IA may be involved only in initiation but not in elongation. Using a template commitment protocol, we have demonstrated that TIF-IA is released from the postinitiation complex and is recruited to preinitiation complexes that lack TIF-IA. Thus, TIF-IA may facilitate reinitiation by association with free Pol I and by converting it into an initiation-competent enzyme. The data presented are consistent with the hypothesis that Pol I and TIF-IA associate to form the transcriptionally active enzyme, defined as the entity which is capable of initiating transcription from the rDNA promoter. This active enzyme is only a minor part of cellular Pol I, i.e., the fraction that is involved in initiation. The vast majority (the bulk enzyme) lacks TIF-IA and, therefore, is unable to catalyze transcription initiation at the rDNA promoter. This view is supported by the observation that functionally different subpopulations of Pol I can be chromatographically separated (35). Furthermore, it has also been reported that the transcriptionally active enzyme is larger than the bulk enzyme (41), an observation which suggests that transcriptionally active Pol I is associated with a protein. By this and other criteria, TIF-IA may be regarded as a functional homolog to the bacterial $\sigma^{70}$ factor or the RNA Pol II-specific transcription factor TFIIF (RAP30/74). Both factors are essential for transcription initiation, associate with their respective core RNA polymerase, and are released by the elongating enzyme. In addition, both $\sigma^{70}$ and TFIIF release RNA polymerase from incorrect start sites and enhance the affinity of the polymerase to the promoter (8, 14, 21).

An activity that is similar if not identical to that of TIF-IA has recently been characterized for a mouse lymphocyte-derived cell line (P1798) that ceases rRNA synthesis when exposed to glucocorticoids (26). Similarly to growth-arrested Ehrlich ascites cells, extracts from hormone-treated lymphosarcoma cells retain the ability to form stable initiation complexes and contain potentially active Pol I but will not initiate unless supplemented with a factor that has been purified from untreated cells. This factor, called TFIC, has been purified extensively, shows no polymerase activity on its own, and exhibits the same functional properties as TIF-IA (16, 25–27). Both factors associate with Pol I and convert stable preinitiation complexes into transcription-competent initiation complexes. Moreover, both TIF-IA and TFIC are required for formation of the first phosphodiester bonds and serve a stoichiometric rather than a catalytic function. In addition, the rate of reinitiation is increased by the amount of TIF-IA and TFIC, a finding which is of particular interest because growth-dependent regulation of cellular rDNA transcription appears to be mediated by changes in the number of productive initiation complexes and not by alterations in the number of open (i.e., transcription-competent) rDNA promoters (11).

The molecular mechanism by which TIF-IA or TFIC augments the rate of reinitiation is not yet known. The observation that the amount of both factors affects the number of rounds of transcription rather than the initial velocity of RNA formation has been interpreted to mean that TIF-I/TFIC may be inactivated as a consequence of initiation (16, 37). This model implies that one molecule of TIF-IA is consumed per initiation event, a hypothesis that we do not consider to be very likely when we take into account the low cellular concentration of this factor and the high rate of cellular pre-rRNA synthesis. Alternatively, after being released from the elongation complex, TIF-IA/TFIC is diluted in the in vitro reaction, and, as pointed out by Gokal et al. (16), inhibition of reinitiation in vitro would therefore be a mass action consequence of a decrease in the physicochemical activity of this factor. None of the data presented in this paper or in the literature enable one to differentiate between these or other hypotheses concerning the apparent stoichiometric requirement for this initiation factor.

The only discrepancy between Thompson's data and our

own concerns the identity of the TIF-IA/TFIC polypeptide(s). Different polypeptides have been observed in the most-purified TIF-IA and TFIC preparations. Although the native molecular mass of TFIC has not been determined, TFIC activity appears to copurify with three polypeptides with estimated molecular masses of approximately 55, 50, and 42 kDa, suggesting that TFIC is a multimeric protein of three subunits. About 20 μg of these polypeptides has been purified from about $8 \times 10^{10}$ cells (26). In contrast, our data demonstrate that TIF-IA activity resides within a single 75-kDa polypeptide which is much less abundant than the polypeptides that copurify with TFIC activity. The following lines of evidence argue against TIF-IA being a multimeric protein. First, the native molecular mass of TIF-IA was determined to be 70 to 80 kDa by size exclusion chromatography on two different columns during different stages of purification. This number corresponds to the molecular mass of the purified protein as estimated by SDS-PAGE and suggests that TIF-IA is a monomeric globular protein. Second, the 75-kDa protein was the only polypeptide that correlated with TIF-IA activity in several factor preparations, irrespective of whether nuclear or cytoplasmic extracts or a mixture of both was used or whether different fractionation schemes were employed. Because of its low cellular abundance, the 75-kDa protein was observed only in late stages of purification. Typically, we started from about $4 \times 10^{11}$ cells (15 g of cell extract proteins) and obtained about 200 ng of the 75-kDa protein. The most-purified TIF-IA preparation was quite unstable, and any subsequent manipulations resulted in total loss of activity. Also, the specific activities of the final TIF-IA fractions were very low. Addition of approximately 0.16 ng (2 fmol) of this protein to the reconstituted transcription system triggers the formation of about 0.15 fmol of initiation complexes, as determined by Sarkosyl inhibition experiments. This number suggests that either 80 to 90% of the 75-kDa protein is transcriptionally inactive or that TIF-IA interacts in a complex stoichiometry with the initiation complex. When the other components were present in excess, there was a linear relationship between the amount of TIF-IA added and the number of initiation complexes formed. Once the system was saturated, about 0.35 fmol of initiation complexes was formed which in turn direct the synthesis of approximately 6 fmol of RNA. Therefore, under optimal conditions, about 20 transcripts are synthesized per active promoter complex in a reconstituted transcription system. However, if TIF-IA was present in excess compared with the number of initiation complexes, the initial velocity of transcription was sustained for longer periods of time. This result, together with those from template commitment experiments, demonstrates that TIF-IA promotes reinitiation of Pol I on stable preinitiation complexes.

The most interesting point with respect to growth-dependent regulation of ribosomal gene transcription is to understand whether the amount or the activity of TIF-IA is modulated according to the proliferation rate of the cell and which molecular mechanism mediates the fluctuations of TIF-IA activity in response to extracellular signals. Answers to these questions will require sufficient amounts of this protein to perform biochemical studies, to clone the cDNA encoding TIF-IA, and to produce antibodies against TIF-IA. This will be a formidable task, if the large quantity of cultured cells required to prepare sufficient amounts of this rare protein to homogeneity is considered. This drawback, together with the fact that TIF-IA binds only to positively charged chromatographic resins, will require the application of other experimental approaches, such as, for instance, the use of a Pol I affinity column, to purify this factor in an amount and degree of purity that are sufficient to study its mode of action.

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## REFERENCES

1. **Arnosti, D. N., A. Merino, D. Reinberg, and W. Schaffner.** 1993. Oct-2 facilitates functional preinitiation complex assembly and is continuously required at the promoter for multiple rounds of transcription. EMBO J. **12**:157–166.
2. **Bateman, E., and M. R. Paule.** 1986. Regulation of eukaryotic ribosomal RNA transcription by RNA polymerase modification. Cell **47**:445–450.
3. **Bell, S. P., H.-M. Jantzen, and R. Tjian.** 1990. Assembly of alternative multiprotein complexes directs rRNA promoter selectivity. Genes Dev. **4**:943–954.
4. **Bell, S. P., C. S. Pikaard, R. H. Reeder, and R. Tjian.** 1989. Molecular mechanisms governing species-specific transcription of ribosomal RNA. Cell **59**:489–497.
5. **Buttgereit, D., G. Plugfelder, and I. Grummt.** 1985. Growth-dependent regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA). Nucleic Acids Res. **13**:8165–8179.
6. **Cavanaugh, A. H., and E. A. Thompson.** 1984. Glucocorticoid inhibition of initiation of transcription of the DNA encoding rRNA (rDNA) in lymphosarcoma P1798 cells. Proc. Natl. Acad. Sci. USA **81**:718–721.
7. **Cavanaugh, A. H., and E. A. Thompson.** 1985. Hormonal regulation of transcription of rDNA: glucocorticoid effects upon initiation and elongation *in vitro*. Nucleic Acids Res. **13**:3357–3369.
8. **Chamberlin, M. J.** 1974. The selectivity of transcription. Annu. Rev. Biochem. **43**:721–775.
9. **Clos, J., D. Buttgereit, and I. Grummt.** 1986. A purified transcription factor (TIF-IB) binds to essential sequences of the mouse rDNA promoter. Proc. Natl. Acad. Sci. USA **83**:604–608.
10. **Comai, L., N. Tanese, and R. Tjian.** 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. Cell **68**:965–976.
11. **Conconi, A., R. M. Widmer, T. Koller, and J. M. Sogo.** 1989. Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. Cell **57**:753–761.
12. **Draetta, G., and D. Beach.** 1988. Activation of cdc2 protein kinase during mitosis in human cells: cell-cycle dependent phosphorylation and subunit rearrangement. Cell **54**:17–26.
13. **Eberhard, D., L. Tora, J. M. Egly, and I. Grummt.** A TBP-containing multiprotein complex (TIF-IB) mediates transcription specificity of murine RNA polymerase I. Nucleic Acids Res., in press.
14. **Flores, O., H. Lu, M. Killeen, J. Greenblatt, Z. F. Burton, and D. Reinberg.** 1991. The small subunit of transcription factor IIF recruits RNA polymerase II into the preinitiation complex. Proc. Natl. Acad. Sci. USA **88**:9999–10003.
15. **Gokal, P., A. H. Cavanaugh, and A. Thompson.** 1986. The effects of cycloheximide upon transcription of rRNA, 5S RNA and tRNA genes. J. Biol. Chem. **261**:2536–2541.
16. **Gokal, P. K., P. B. Mahajan, and A. Thompson.** 1990. Hormonal regulation of rDNA. Formation of initiated complexes by RNA polymerase I *in vitro*. J. Biol. Chem. **265**:16234–16243.
17. **Grummt, I., E. Roth, and M. Paule.** 1982. Ribosomal RNA transcription *in vitro* is species-specific. Nature (London) **296**:173–174.
18. **Grummt, I., V. A. Smith, and F. Grummt.** 1976. Amino acid

starvation affects the initiation frequency of nucleolar RNA polymerase. Cell **7**:439–445.
19. **Hammond, M. L., and L. H. Bowmann.** 1988. Insulin stimulates the translation of ribosomal proteins and the transcription of rDNA in mouse myoblasts. J. Biol. Chem. **263**:17785–17791.
20. **Hawley, D. K., and R. G. Roeder.** 1987. Functional steps in transcription initiation and reinitiation from the major late promoter in HeLa nuclear extract. J. Biol. Chem. **262**:3452–3461.
21. **Killeen, M. T., and J. F. Greenblatt.** 1992. The general transcription factor RAP30 binds to RNA polymerase II and prevents it from binding nonspecifically to DNA. Mol. Cell. Biol. **12**:30–37.
22. **Kuhn, A., and I. Grummt.** 1992. Dual role of the nucleolar transcription factor UBF: trans-activator and antirepressor. Proc. Natl. Acad. Sci. USA **89**:7340–7344.
23. **Kuhn, A., V. Stefanofsky, and I. Grummt.** 1993. The nucleolar transcription factor UBF relieves Ku antigen-mediated repression of mouse ribosomal gene transcription. Nucleic Acids Res. **21**:2057–2063.
24. **Learned, M. R., S. Cordes, and R. Tjian.** 1985. Purification and characterization of a transcription factor that confers promoter specificity to human RNA polymerase I. Mol. Cell. Biol. **5**:1358–1369.
25. **Mahajan, P. B., P. K. Gokal, and A. E. Thompson.** 1990. Hormonal regulation of transcription of rDNA. The role of TFIC in formation of initiation complexes. J. Biol. Chem. **265**:16244–16247.
26. **Mahajan, P. B., and A. E. Thompson.** 1990. Hormonal regulation of transcription of rDNA. Purification and characterization of the hormone-regulated transcription factor IC. J. Biol. Chem. **265**:16225–16233.
27. **Mahajan, P. B., and A. E. Thompson.** 1992. Copurification of RNA polymerase I and the glucocorticoid-regulated transcription factor IC. Protein Expression Purif. **3**:410–416.
28. **McStay, B., C. H. Hu, C. S. Pikaard, and R. H. Reeder.** 1991. xUBF and Rib 1 are both required for formation of a stable polymerase I promoter complex in *X. laevis*. EMBO J. **10**:2297–2303.
29. **Mishima, Y., T. Matsui, and M. Muramatsu.** 1979. The mechanism of decrease in nucleolar RNA synthesis by protein synthesis inhibition. J. Biochem. **85**:807–818.
30. **Paule, M. R.** 1993. Polymerase I transcription, termination, and processing. Gene Expr. **3**:1–9.
31. **Paule, M. R. (Colorado State University).** 1993. Personal communication.
32. **Paule, M. R., C. T. Iida, P. J. Perna, G. H. Harris, D. A. Knoll, and J. M. D'Alessio.** 1984. *In vitro* evidence that eukaryotic ribosomal RNA transcription is regulated by modification of RNA polymerase I. Nucleic Acids Res. **12**:8161–8180.
33. **Schnapp, A., and I. Grummt.** 1991. Transcription complex formation at the mouse rDNA promoter involves the stepwise association of four transcription factors and RNA polymerase I. J. Biol. Chem. **266**:24588–24595.
34. **Schnapp, A., C. Pfleiderer, H. Rosenbauer, and I. Grummt.** 1990. A growth-dependent transcription initiation factor (TIF-IA) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. EMBO J. **9**:2857–2863.
35. **Schnapp, G., and I. Grummt.** Unpublished data.
36. **Smid, A., M. Finsterer, and I. Grummt.** 1992. Limited proteolysis unmasks specific DNA-binding of the murine RNA polymerase I-specific transcription termination factor TTFI. J. Mol. Biol. **227**:635–647.
37. **Sollner-Webb, B., L. Pape, K. Ryan, E. B. Mougey, R. Poretta, E. Nikolow, M. H. Paalmann, I. Lazdins, and C. Martin.** 1989. Expression of mouse and frog rRNA genes: transcription and processing. Mol. Cell. Biochem. **104**:149–154.
38. **Surmacz, E., L. Kaczmarek, and R. Baserga.** 1987. Activation of the ribosomal DNA promoter in cells exposed to IGF-1. Mol. Cell. Biol. **7**:657–663.
39. **Tanaka, N., H. Kato, Y. Ishikawa, K. Hisatake, K. Tashiro, K. Kominami, and M. Muramatsu.** 1990. Sequence-specific binding of a transcription factor TFID to the promoter region of mouse ribosomal RNA gene. J. Biol. Chem. **265**:13836–13842.
40. **Tower, J., V. Culotta, and B. Sollner-Webb.** 1986. Factors and nucleotide sequences that direct ribosomal DNA transcription and their relationship to the stable transcription complex. Mol. Cell. Biol. **6**:3451–3462.
41. **Tower, J., and B. Sollner-Webb.** 1987. Transcription of mouse rDNA is regulated by an activated subform of RNA polymerase I. Cell **50**:873–883.
42. **Zieve, G. W., D. Turnbull, J. M. Mullins, and J. R. McIntosh.** 1980. Production of large number of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Exp. Cell Res. **126**:397–405.