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Growth-dependent regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA)

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ABSTRACT

Mouse RNA polymerase I requires at least two chromatographically distinct transcription factors (designated TIF-IA and TIF-IB) to initiate transcription accurately and efficiently in vitro. In this paper we describe the partial purification of TIF-IA by a four-step fractionation procedure. The amount or activity of TIF-IA fluctuates in response to the physiological state of the cells. Extracts from quiescent cells are incapable of specific transcription and do not contain detectable levels of TIF-IA. Transcriptionally inactive extracts can be restored by the addition of TIF-IA preparations that have been highly purified from exponentially growing cells. During the fractionation procedure TIF-IA co-purifies with RNA polymerase I, suggesting that it is functionally associated with the transcribing enzyme. We suggest that only those enzyme molecules that are associated with TIF-IA are capable to interact with TIF-IB and to initiate transcription.

INTRODUCTION

The transcription of the genes encoding the major rRNA species is very efficiently regulated and is altered in response to the physiological state of the cells. It has been well documented that nutrient starvation (1), hormone administration (2 - 4) and viral infection (5, 6) affects the transcription of ribosomal genes. The molecular mechanism by which this regulation is brought about is poorly understood. Early investigations using isolated nucleoli suggested that the efficiency of rDNA transcription is regulated by the initiation frequency of RNA polymerase I. It has been postulated that this regulation is mediated by a protein with a high turnover rate (1, 4, 7). The development of cell-free systems that faithfully initiate transcription on cloned rDNA has proved to be useful for mapping essential polymerase I promoter elements (8 - 15) and fa-

cilitates a biochemical analysis of the transcription machinery. Interestingly, the polymerase I transcription system mimics the in vivo control. We could show that extracts from exponentially growing cells are much more active in rDNA transcription than are extracts from stationary cells responding to nutritional shift-down (16). This alteration in the transcriptional activity could be due to a modification of the RNA polymerase I (17) or due to changes in the levels or activity of regulatory factor(s) that are required for efficient initiation.

In this study we tried to distinguish between these two possibilities. In particular, we attempted to purify the component(s) that is responsible for the modulation of the initiation frequency of RNA polymerase I. Using a four-step fractionation procedure we could partially purify a regulatory transcription initiation factor (TIF-IA), the amount or activity of which fluctuates according to the proliferation rate of the cells. This initiation factor obviously responds to changes in the extracellular environment, thus mediating the level of rDNA transcription according to the proliferation rate of the cells.

MATERIALS AND METHODS

Preparation of transcriptionally active and inactive extracts

For the preparation of transcriptionally active extracts Ehrlich ascites tumor cells were cultured in RPMI medium in the presence of 5 % newborn calf serum for at least 24 h and harvested at a density of 8×10^5 cells/ml. The preparation of S100 extracts occurred according to Weil et al. (18), nuclear extracts were made according to Dignam et al. (19). Transcriptionally inactive extracts that can be complemented by partially purified TIF-IA preparations were usually derived from cells grown in the abdominal cavity of the mouse at a density of 1×10^8 cells per ml ascites fluid. Alternatively, cells were cultured for 3 - 4 hours in serum-free medium at 5×10^7 cells/ml.

Specific transcription assay

5 - 15 µl of extract or column fractions were assayed in a total volume of 25 µl containing 12 mM Hepes (pH 7.9), 85 mM

KCl, 0.12 mM EDTA, 0.5 mM DTE, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 mM each of ATP, CTP, and UTP, 12.5 µM GTP, and 2 µCi α-^{32}P GTP (spec. act. 400 Ci/mmol) and 20 - 100 ng of template DNA pMr600/Eco RI (20). After incubation for 45 min at 30° C the specific runoff transcript (297 nucleotides) was analyzed by electrophoresis on a 4 % polyacrylamide gel.

RNA polymerase I assay

Random transcription assays contained 25 mM Tris (pH 7.9), 5 mM $MgCl_2$, 0.6 mM of the three unlabelled ribonucleoside triphosphates, 1 µCi ^3H-UTP, 200 µg/ml α-amanitin and column fractions in a total volume of 25 µl. Samples were incubated for 20 min at 37° C, precipitated with trichloroacetic acid, and the acid-insoluble radioactivity was measured.

Measurement of rRNA synthesis in permeabilized cells

5×10^6 Ehrlich ascites cells were spun down, washed in PBS and resuspended in 100 µl PBS containing 0.05 % NP 40. rDNA transcription was measured in 5 µl of these permeabilized cells in a 50 µl assay containing 50 mM Hepes (pH 7.8), 5 mM $MgCl_2$, 2 mM DTT, 75 mM KCl, 0.5 mM of the unlabelled nucleoside triphosphates, 1.5 µCi ^3H-UTP, 0.01 mM UTP and 200 µg/ml α-amanitin. After incubation for 15 min at 30° C the acid-precipitable radioactivity was determined.

Assay for TIF-IA activity

Individual column fractions were tested for their ability to reconstitute specific runoff RNA synthesis in transcriptionally inactive extracts derived from quiescent cells. For this, 7.5 µl of inactive extract and 7.5 µl of column fractions which on their own are not capable of accurate transcription initiation were incubated under standard reaction conditions and processed for gel analysis as described before (16). The amounts of transcripts were quantitated by densitometric scanning of the autoradiograms. Comparison with the specific transcription directed by extracts from exponentially growing cells yields some estimates for the specific activity of TIF-IA during the purification.

Purification of TIF-IA activity

The fractionation scheme for the purification of TIF-IA is shown in Fig. 4 and 5. A mixture of S100 and nuclear extracts

derived from cultured cells (250 ml) was applied onto a 500 ml DEAE-Sephadex column equilibrated with buffer A (20 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTE, 20 % glycerol) containing 0.1 M KCl (buffer A-100). The column was washed with two volumes of A-100 and the active fractions were eluted with 200 mM KCl. Fractions from the DE200 step were applied directly to a Heparin-Ultrogel column, equilibrated with buffer A-200 (column volume 50 ml). The column was washed with two volumes of A-200 and bound protein was eluted with three salt steps containing 400, 600 and 1,000 mM KCl. The fractions eluting at 400 mM KCl were pooled, diluted to 100 mM KCl and loaded onto a 10 ml Blue Sepharose column that had been equilibrated with buffer A-100. The column was washed with two volumes of the same buffer and eluted with 300 and 500 mM KCl steps. The RNA polymerase I and TIF-IA containing fractions, eluting at 300 mM KCl, were diluted to 100 mM KCl and chromatographed on a 2 ml Bio-Rex 70 column. After washing the column with two volumes of buffer A-100 two salt steps were applied (300 and 500 mM KCl) to elute TIF-IA and RNA polymerase I activity.

RESULTS

The cell-free transcription system reflects the rRNA synthetic activity of the cells

After nutritional shift-down the transcription of the ribosomal genes rapidly decreases to 20 - 25 % of the rate of exponentially growing cells (1, 21 and Fig. 1 A). In order to study the molecular mechanisms which regulate rRNA synthesis according to the physiological state of the cells, we have used a cell-free system which faithfully initiates transcription on pMr600, a plasmid which contains the 5' end of the mouse rDNA transcription unit (16, 20). Interestingly, this RNA polymerase I-specific in vitro system mimics the control of rRNA synthesis observed *in vivo*. As shown in Fig. 1 B (lane a) extracts prepared from exponentially growing Ehrlich ascites cells support high levels of specific rDNA transcripts. Extracts from cells harvested after nutritional shift-down or from stationary cells are virtually inactive (lane b). Complementation of these transcriptionally inactive extracts with active extracts from

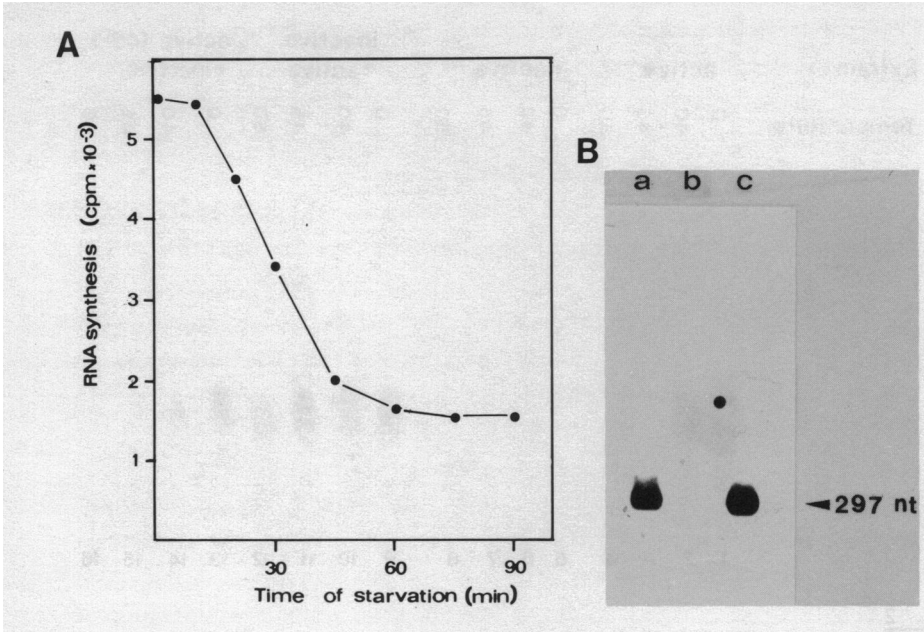

Fig. 1
Response of rRNA synthesis to nutrional shift-down
A) Ehrlich ascites cells growing in suspension culture at a density of 5 x 10^6 cells/ml were transferred to histidine-free RPMI medium. At different time points 10 ml aliquots of the cells were spun down, suspended in 100 µl PBS containing 0.05 % NP 40 and cellular rRNA synthesis was measured as described in Materials and Methods.
B) Activity of extracts from logarithmically growing and starved cells in the cell-free rDNA transcription system. Extracts from exponentially growing cells (lane a), extract from cells that have been starved for serum for 3 hrs (lane b), mixture of equal amounts of each extract (lane c).

growing cells restores synthetic activity (lane c). When assayed for protein concentration and RNA polymerase I activity both extracts from growing and quiescent cells had approximately the same protein and RNA polymerase I content (not shown). This indicates that the limiting component for specific transcription is not the availability of RNA polymerase I but some initiation factor(s), designated TIF-IA, that is missing in the starved cells.

TIF-IA is less thermolabile than RNA polymerase I

RNA polymerase I has been reported to be extremely thermolabile (21), exhibiting a half-time of denaturation at 45° C of

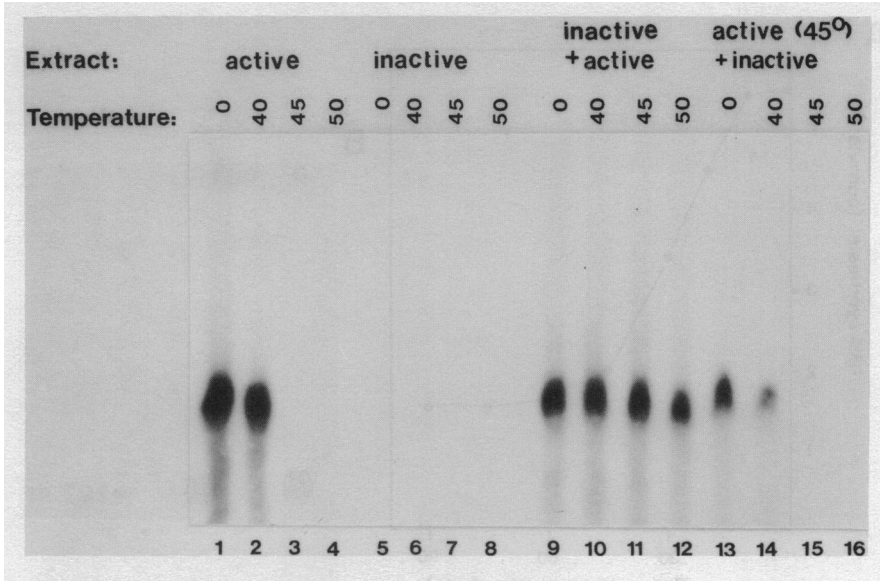

Fig. 2
Temperature sensitivity of RNA polymerase I and TIF-IA in active and inactive extracts. The extracts were incubated for 15 min at the specified temperature. Lanes 1 - 4: active extract from growing cells, lanes 5 - 8: inactive extracts from quiescent cells, lane 9 - 12: inactive extract, complemented with active heat-treated extract, lanes 13 - 16: active extract, incubated at 45° C and complemented with the inactive heat-treated extract.

about 1 minute. In order to investigate whether TIF-IA shows a different thermosensitivity than polymerase I a crude extract was incubated at various temperatures between 40 and 50° C before specific rDNA transcription was assayed. As shown in Fig. 2 incubation of the extract for 15 min at 45° C inactivates the polymerase I which results in a complete loss of specific transcription. If, however, a transcriptionally inactive extract from starved cells was supplemented with the heat-treated extracts, which showed no residual RNA polymerase I activity, transcriptional activity was fully restored. This finding shows that the initiation factor TIF-IA, which is capable of reconstituting transcriptional activity in extracts from growth-inhibited cells is less heat-sensitive than RNA polymerase I and survives preincubation at temperatures up to 50° C.

A control experiment was carried out in which both the active

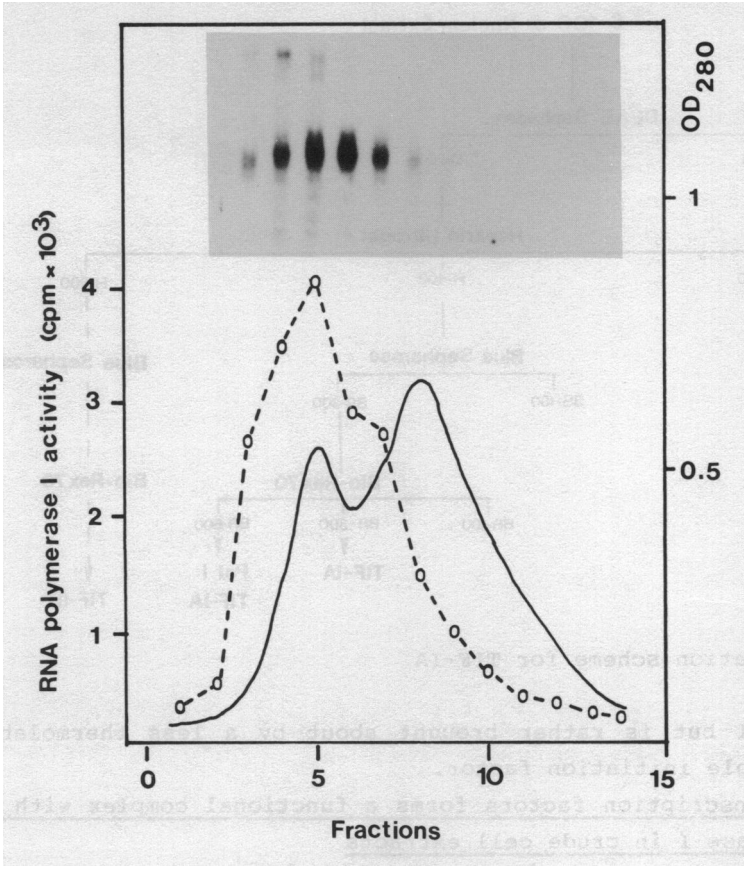

Fig. 3
Gel filtration of a nuclear extract on Trisacryl GF 2000
30 µl of active extract were applied onto a 18 x 0.3 cm column. Elution was performed with buffer A-100 at 1.2 ml/hr. Fractions of 60 µl were collected and assayed for RNA polymerase I activity with nicked calf thymus DNA (o---o) and for specific transcription in the runoff assay (inset). The maximum of RNA polymerase I activity and specific transcription elutes at a molecular weight of approximately 5 x 10^5 Dalton.

and inactive extract was heated at 45° C before mixing and incubation for *in vitro* transcription. As shown in Fig. 2, lanes 15 and 16, this mixture did not synthesize the 297 nucleotide RNA. The heat-inactivation profile of RNA polymerase I in active and inactive extracts is absolutely identical (unpublished observations). Thus it appears that the difference in the transcriptional activitiy of extracts from growing and non-growing cells is not caused by a modification of the RNA poly-

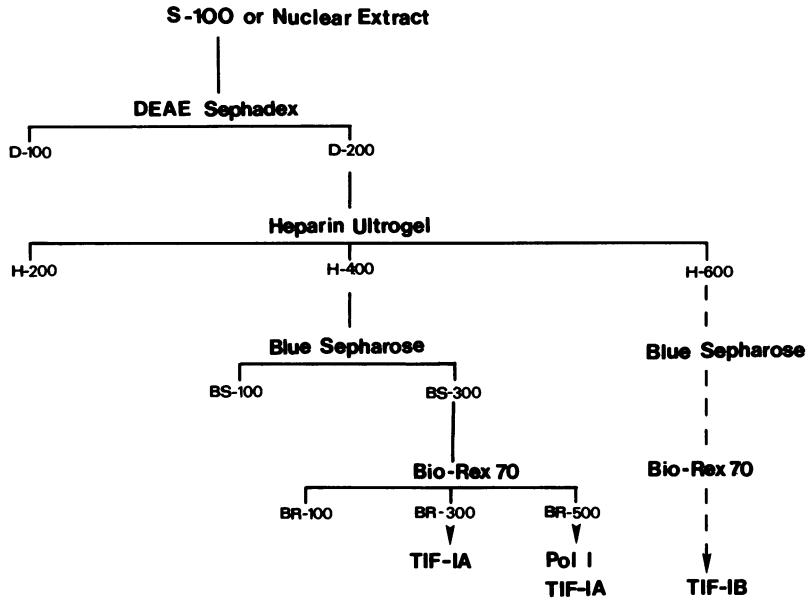

Fig. 4
Purification scheme for TIF-IA

merase I but is rather brought about by a less thermolabile diffusable initiation factor.

The transcription factors forms a functional complex with RNA polymerase I in crude cell extracts

Recently it has been shown that RNA polymerase II and transcription initiation factors are not associated in crude cell extracts but assemble into transcription initiation complexes after addition of specific promoter sequences (22). In order to investigate whether in active extracts RNA polymerase I is already associated with the essential initiation factors, a nuclear extract was fractionated by gel filtration on Trisacryl GF2000. The column fractions were assayed both for the activity of polymerase I on nicked calf thymus DNA in the presence of 200 µg/ml α-amanitin and for the ability to direct specific transcription initiation on the linearized rDNA plasmid. As shown in Fig. 3 the distribution of specific transcriptional activity was similar to the distribution of RNA polymerase I activity. No specific transcription can be detected in the fractions containing RNA polymerase I activity after sizing of

a nuclear extract from starved cells. This association of transcription factors with the RNA polymerase I is still detectable after running the column at 1 M KCl, rendering unlikely that these polymerase-factor complexes represent fortuitous aggregates.

These results together with the extensive copurification described below suggest that in transcriptionally active cell-free extracts RNA polymerase I and the transcription initiation factors preexist as a functional complex.

Partial purification of pol I transcription initiation factors

In order to isolate the factors required for rDNA transcription initiation, mixtures of S100 and nuclear extracts were subjected to a multi-step fractionation procedure. The scheme adopted for partial purification of mouse rDNA transcription factors is shown in Fig. 4. The first step involved chromatography on DEAE-Sephadex (Figure 5 A). The fractions eluting at 200 mM KCl contained the RNA polymerase I activity and, by itself, supported accurate transcription of rDNA, indicating that RNA polymerase I and the transcription factors copurify on DEAE-Sephadex. The active fractions were further purified on Heparin-Ultrogel (Fig. 5 B). This chromatographic step separated RNA polymerase I from another essential transcription initiation factor TIF-IB. All of the RNA polymerase I activity was eluted from the heparin column by a 0.2 - 0.4 M KCl salt step (fraction H-400), but this fraction alone was unable to accurately initiate RNA synthesis from the rDNA promoter. Addition of proteins eluting at 0.6 M KCl (fraction H-600) to the polymerase I containing fractions restored the accuracy of transcription, indicating that components that impart selectivity to RNA polymerase I were separated from the enzyme.

Next we investigated whether the regulatory factor, the presence or activity of which fluctuates according to the proliferation rate of the cells, was contained in the H-400 or H-600 fraction. For this, transcriptionally inactive extracts derived from starved or stationary cells were subjected to the same fractionation procedure on DEAE-Sephadex and on Heparin-Ultrogel as outlined above. No differences in the distribution of protein and the specific activity of RNA polymerase I could

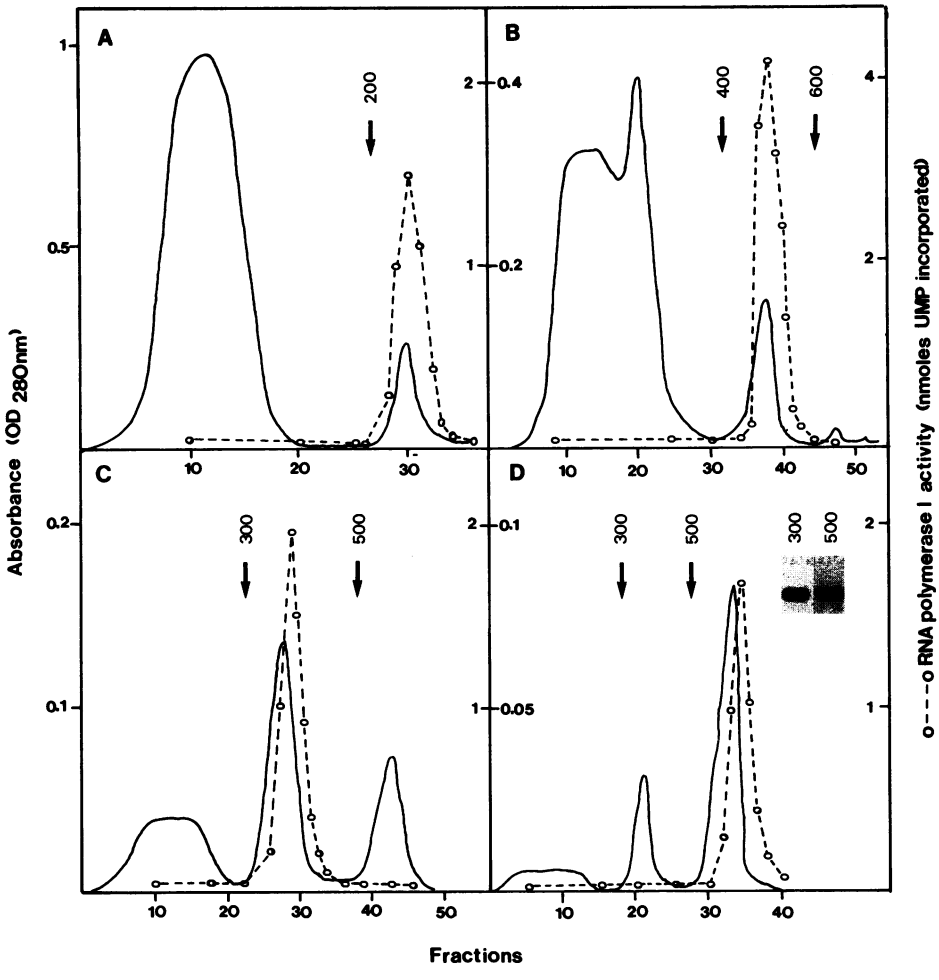

Fig. 5
Column chromatography of TIF-IA
A) DEAE-Sephadex, B) Heparin-Ultrogel, C) Blue Sepharose, D) Bio-Rex 70. RNA polymerase I and TIF-IA activities were measured as described in Materials and Methods. In panels A to C TIF-IA activity coincides with RNA polymerase I activity. The inset in panel D shows that on Bio-Rex 70 TIF-IA activity elutes at 300 and 500 mM KCl. The position of the salt steps applied is marked by arrows, the numbers above the arrows indicate the concentration of salt (mM KCl). The amount of proteins applied onto the DEAE column was 5 grams. The final yield after chromatography on Bio-Rex 70 was 0.06 mg protein in the BR-300 fraction and 0.15 mg in the BR-500 fraction.

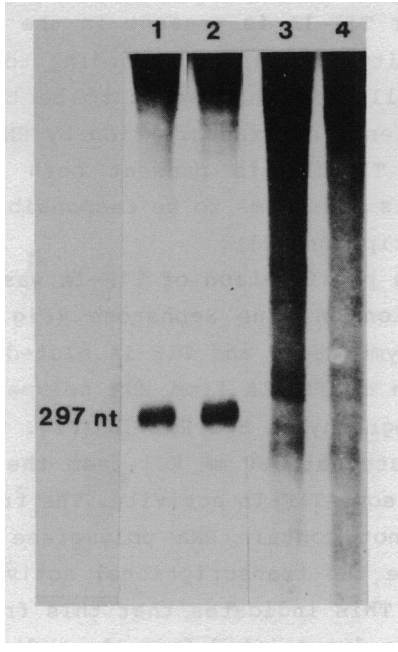

Fig. 6
Heterologous reconstitution of H-400 and H-600 fractions
3 µl of each fraction were used in the transcription assay. Lane 1) fraction H-400 derived from active extract + H-600 from active extract, lane 2) H-400 from active extract + H-600 from inactive extract, lane 3) H-400 from inactive extract + H-600 from active extract, lane 4) H-400 from inactive extract + H-600 from inactive extract.

be detected as compared to the active extracts. However, none of the DEAE- or Heparin fractions alone or in combination directed the synthesis of specific run-off transcripts.
Functional differences between transcriptionally active and inactive extracts are probably restricted to the H-400 fraction. Reconstitution experiments with fractions H-400 and H-600 from both types of extracts showed, that only the fraction H-400 from active extracts was able to restore specific initiation (Fig. 6). On the contrary, fractions H-600 both from transcriptionally active and inactive extracts were equally capable to restore selective transcription of a H-400 fraction derived from active extracts. We interpret this result to mean, that in addition to RNA polymerase I at least two more factors are required for accurate and efficient transcription initia-

tion on rDNA. Factor TIF-IA is present in the fraction H-400. Its amount or activity fluctuates according to the proliferation rate of the cells and thus seems to be the protein that regulates the efficiency of transcription by RNA polymerase I. The second factor, TIF-IB, is present both in growing and growth-arrested cells and seems to be responsible for the accuracy of rDNA transcription (23).

The next step in the purification of TIF-IA was chromatography of the H-400 fraction on Blue Sepharose (Fig. 5 C). On this column both RNA polymerase I and TIF-IA eluted at 300 mM KCl. Partial dissociation of TIF-IA from RNA polymerase I could be obtained by chromatography on Bio-Rex 70 (Fig. 5 D). All of the RNA polymerase I eluted at 500 mM KCl from the Bio-Rex 70 column, together with some TIF-IA activity. The fractions eluting at 300 mM KCl did not contain RNA polymerase I activity but were able to restore the transcriptional activity of extracts from starved cells. This indicates that this fraction contains TIF-IA which has been dissociated from the endogenous RNA polymerase I on Bio-Rex 70. Although TIF-IA was purified through four chromatographic steps at this stage, we have not yet been able to identify the TIF-IA protein by correlating TIF-IA activity with a distinct polypeptide or a set of polypeptides. Moreover, due to the complexity of the transcription reaction and the lability of TIF-IA at later stages of purification it is difficult to quantitate the yield and the degree of purification through the various fractionation steps. Estimates for the specific activity of TIF-IA so far are based on a titration of the amount of protein required to supplement trancriptionally inactive extracts to obtain the same amount of specific transcript as in extracts from rapidly growing cells. Based on this measurements the specific activity of TIF-IA increased about 2000-fold during the procedure described. However, a less ambiguous determination of the cellular content of TIF-IA will finally require the use of antibodies against the factor.

DISCUSSION

We have investigated the molecular mechanisms that regulate rRNA synthesis according to the proliferation rate of the

cells. Previously we have reported that the transcriptional activity of extracts prepared from growing or quiescent Ehrlich ascites cells was drastically different (16) and concluded that the intracellular level of a transcription initiation factor may be modulated in order to regulate the transcriptional activity of rRNA genes. The present studies support this conclusion. We show that initiation per se requires at least two additional factors, called TIF-IA and TIF-IB. TIF-IB is a specific DNA-binding protein that interacts with the core element of the mouse rDNA promoter and is described elsewhere (23). The assayable levels of TIF-IB and RNA polymerase I are virtually identical in rapidly or slowly growing cells. The other factor TIF-IA seems to be the target for rDNA transcription regulation. Addition of partially purified TIF-IA to transcriptionally inactive extracts from quiescent cells restored transcriptional activity. This result is interpreted to mean that the failure of initiation in extracts from growth-inhibited cells is due to depletion of active TIF-IA and is not attributable to accumulation of some inhibitior(s) that counteract TIF-IA.

There is an apparent inconsistency between the transcriptional activity of resting cells and the cell-free system. The rate of ribosomal RNA synthesis after nutrient starvation is still 20 - 30 % as compared to the transcription rate of rapidly growing cells. Yet extracts prepared from quiescent cells fail to direct any specific transcription in vitro. This discrepancy may be due to the fact that the transcriptional activity in the cell-free system shows a sigmoidal dose dependence (data not shown), indicating that multiple protein factors are involved in the initiation reaction. Thus a threshold concentration of the individual factors is required to observe any transcription at all. Apparently the concentration of factor TIF-IA in extracts from resting cells is below this threshold.

In crude extracts the RNA polymerase I seems to be associated with the essential transcription factors. This functional association proves to be rather tight since the complex consisting of RNA polymerase I and the factors TIF-IA and TIF-IB still exists after treatment of the extracts with 1 M KCl, precipitation with 40 % ammonium sulphate or chromatography on DEAE-

Sephadex. TIF-IB is dissociated from the complex by chromatography on Heparin-Ultrogel. This finding is in contrast to results from Kurl et al. (24) who reported on the fractionation of transcription factors from rat nuclear extracts. These authors did not resolve the factors by chromatography on Heparin-Sepharose, using a rather steep salt gradient. In our hands, the RNA polymerase I enriched fraction (H-400) contains the TIF-IA activity without any TIF-IB. TIF-IA copurifies with the RNA polymerase I on four different columns (DEAE, heparin, phosphocellulose, Blue Sepharose) and can be only partially separated from RNA polymerase I on Bio-Rex 70. At present we cannot decide whether the copurification of TIF-IA and RNA polymerase I occurs fortuitiously, or whether TIF-IA is functionally associated with a subpopulation of RNA polymerase I molecules.

Our working hypothesis is that TIF-IA is associated with the RNA polymerase I. Only those polymerase I molecules that have bound TIF-IA are able to recognize and interact with TIF-IB, the protein that is responsible for the formation of stable preinitiation complexes with the rDNA promoter. This hypothesis is supported by results of Cavanaugh et al. (25). These authors have shown that glucocorticoid-mediated inhibition of proliferation of lymphosarcoma P1798 cells in culture does neither affect the levels of polymerase I, the elongation rate of transcription, nor the rates of stable complex formation. Initiation of rDNA transcription in extracts from hormone-treated cells could be restored by the addition of a phosphocellulose fraction from control cells, which was designated TFIC according to the convention of Mishima et al. (26). TFIC is probably identical to our TIF-IA factor.

Recently Paule et al. (17) provided evidence that down-regulation of rRNA transcription during encystment of Acanthamoeba castellanii is caused by some modification of RNA polymerase I. In this differentiating system a subtle alteration of the RNA polymerase I leads to the loss of its ability to faithfully initiate RNA chains at the rDNA promoter. RNA polymerase I purified from cysts is significantly less able to complement the inactive extracts than is polymerase I purified from vegeta-

tively growing cells. Thus it appears that there may be several distinct mechanisms for regulating rRNA transcription in different eukaryotic cell types and in different regulatory situations. The fluctuations in the level of TIF-IA in response to physiological variations could be caused by phosphorylation, adenylylation or similar modifications of the protein. Such a modification of the TIF-IA would permit rapid alterations in the rate of rRNA synthesis without recourse to synthesis of new protein.

In this context it is interesting that the intracellular levels of GTP and ATP have been shown to directly affect the initiation frequency of RNA polymerase I, even in the presence of protein synthesis inhibitors (27). Further purification of TIF-IA and the use of antibodies will be necessary to carry out more detailed biochemical analysis and to find out whether the changes of TIF-IA activity are mediated by modification of this transcription factor.

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