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In vitro evidence that eukaryotic ribosomal RNA transcription is regulated by modification of RNA polymerase I

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ABSTRACT

We have utilized a cell-free transcription system from Acanthamoeba castellanii to test the functional activity of RNA polymerase I and transcription initiation factor I (TIF-I) during developmental down regulation of rRNA transcription. The results strongly suggest that rRNA transcription is regulated by modification, probably covalent, of RNA polymerase I: (1) The level of activity of TIF-I in extracts from transcriptionally active and inactive cells is constant. (2) The number of RNA polymerase I molecules in transcriptionally active and inactive cells is also constant. (3) In contrast, though the specific activity of polymerase I on damaged templates remains constant, both crude and purified polymerase I from inactive cells have lost the ability to participate in faithful initiation of rRNA transcription. (4) Polymerase I purified from transcriptionally active cells has the same subunit architecture as enzyme from inactive cells. However, the latter is heat denatured 5 times faster than the active polymerase.

INTRODUCTION

In eukaryotic systems, three components are necessary for faithful initiation of transcription in vitro: (1) a DNA template containing the primary sequence elements specifying the start site for transcription, (2) one of the DNA-dependent RNA polymerases and (3) a third component or group of components known as transcription initiation factors (TIF). There are three distinct RNA polymerases in eukaryotic cells, each of which transcribes a separate group of genes: polymerase I transcribes ribosomal RNA precursor, the class II polymerase transcribes protein genes and, polymerase III transcribes pre tRNA, 5S RNA and a few other RNA species. The subunit compositions of the polymerases reflect these distinct functions: each enzyme consists of a group of subunits unique to the polymerase class in addition to a group of subunits common to all three polymerases (1-3). Similarly, there are a separate set of TIFs for each RNA polymerase. In the case of the polymerase III factors, there are both TIFs globally used in transcription of all class III-transcribed genes and distinct factors

specifically involved in transcription of particular class III genes (4,5). Evidence for a similar set of specific and general TIFs for class II genes has been reported recently (6). The identification of transcription unit-specific factors leads to the speculation that the expression of a given gene could be regulated by the availability of its specific TIF. This type of mechanism is particularly appealing for 5S RNA which has been shown to have a TIF dedicated solely to 5S transcription. Indeed, Honda and Roeder (7) and Pelham and Brown (8) have presented evidence supporting the notion that the availability of the 5S RNA-specific TIF (TF IIIA) may play some role in the regulation of 5S RNA synthesis in developing Xenopus laevis oocytes, though the exact mechanism is complex (9).

The precursor ribosomal RNA genes of eukaryotic cells are ideally suited for this type of regulatory scheme as well. They are expressed by a polymerase-TIF pair solely dedicated to their synthesis. Thus, like the 5S RNA genes, rRNA synthesis could readily be regulated by alteration of the levels of the specific transcription initiation factor(s) (TIF-I). Grummt (10) has reported that crude TIF-I preparations from rapidly-growing mouse Ehrlich ascites cells are very active in promoting faithful in vitro transcription of mouse rRNA while similar extracts from slowly-growing ascites cells are inactive in vitro. Since the rate of rRNA transcription is greatly reduced in the slowly growing cells, the transcription factor activity of these extracts roughly parallels the rate of rRNA transcription in these cells and prompted the suggestion that the level of TIF-I may indeed regulate rRNA transcription in the mouse. We examined the universality of this suggestion and show here that the level of TIF-I does not parallel the activity of the rRNA genes in a differentiating eukaryotic protist.

Since the only function of RNA polymerase I in the cell is the transcription of rRNA, gene regulation could equally be accomplished by altering the level of active RNA polymerase I in the cell. Some studies have shown changes in the levels of RNA polymerase I in cells undergoing unusually rapid growth. However, the bulk of evidence suggests that this is not a primary regulatory mechanism for rRNA gene expression. In cells undergoing normal growth there is an invariant basal level of RNA polymerase I despite wide fluctuations in rRNA transcriptional activity (11,12). All of the studies reported to date have estimated the number of active RNA polymerase molecules in cellular extracts by measuring the synthesis of RNA using highly damaged nuclear DNA. These templates are effective for

measurement of chain elongation, but due to the preponderance of nonspecific initiations at nicks, single-stranded gaps and "frayed" ends, they cannot be used to measure more subtle changes in the polymerase which might effect promotor recognition and initiation events. Indeed, using this nonspecific assay system we have found that the number of polymerase I molecules remains constant in Acanthamoeba castellanii cells during developmentally regulated shutoff of rRNA synthesis (11). Subsequently, however, we developed an in vitro transcription system which allows estimation of the number of RNA polymerase I molecules which are capable of faithful and efficient initiation of rRNA gene transcription at the rRNA promoter. Using this initiation assay, we show here that the ability of polymerase I to specifically initiate synthesis of rRNA is lost in parallel with in vivo shutoff of the transcription unit. Since this loss of ability to initiate transcription is retained in RNA polymerase I after purification to homogeneity, these results are strong evidence that rRNA transcription is regulated by polymerase modification.

METHODS

Growth and encystment of cells

Acanthamoeba castellanii were grown in the proteose peptone-yeast extract-glucose medium of Neff as previously described (13) and synchronous encystment was induced by transfer to Neff's constant-pH encystment medium as previously described (13).

Pulse labeling of RNA

To cells in 15 ml of constant-pH encystment medium in 50 ml erlenmeyer flasks, 150 µCi of 5-[^{3}H]-uridine (>30 Ci/mmole,New England Nuclear) were added at selected times after transfer to encystment medium. At the termination of a 1 hr pulse, cells were collected by centrifugation at 4°C. The supernatants were removed and the cells lysed by the addition of 3 ml of 300 mM NaCl, 2 mM aurintricarboxylic acid, 10 mM Tris-Cl, pH 7.2, 0.5% sodium dodecylsulfate, 0.1 mM $MgCl_2$ (extraction buffer). Following extraction once with phenol and 3 times with chloroform-isoamyl alcohol (100:1), both saturated with extraction buffer, the RNA was precipitated overnight at -20°C with 2 volumes of ethanol. Precipitated RNAs were collected by centrifugation at 30,000 RPM, 4°C for 30 min in a Beckman SW 50.1 rotor. Pellets were dissolved in 150 µl of 300 mM NaCl, 10 mM EDTA, 10 mM Tris-Cl, pH 7.2, 0.5% sodium dodecylsulfate and the RNAs separated by rate zonal sedimentation. 5% - 29.1% sucrose isokinetic gradients in the

Beckman SW41 rotor were centrifuged for 5.5 hr at 41,000 RPM, 20°C and fractionated using an ISCO density gradient fractionator. Absorbance was monitored at 254 nm with an ISCO UA-5 absorbance monitor. One hundred µl aliquots from fractions 13 through 30 of each gradient were counted in 10 ml of Triton-toluene scintillant [33% (v/v) Triton X100, 60% (v/v) toluene containing 14.9 g PPO and 0.3 g Bis-MSB per gallon]. Radioactivity was corrected for differences in the yield of RNA by normalizing to the integrated areas under the 18S and 26S absorbance peaks.

Preparation of S100 extracts and in vitro transcription assays

S100 extracts of vegetative cells or encysting cells were prepared by the method of Weil et al. (14). The extracts were partially purified by chromatography on phosphocellulose (P11, Whatman). S100 (15ml) dialyzed against Buffer A (20 mM Tris-Cl pH 7.9, 0.2mM EDTA, 1mM dithiothreitol, 20% (v/v) glycerol) containing 0.1M KCl was applied to a phosphocellulose column (2.5 x 5.0 cm) equilibrated with 0.1M KCl in Buffer A. The column was washed with 5 volumes of Buffer A containing 0.1M KCl, then fractionated with 3 volumes of Buffer A containing 0.4M KCl, 3 volumes of Buffer A containing 0.6M KCl and 3 volumes of Buffer A containing 1.0M KCl. Fractions were pooled according to absorbance at 280 nm, dialyzed at 4°C for 4 hours against Buffer A containing 0.1M KCl, and assayed for TIF and RNAP I activity. The 0.6M KCl fraction (designated TIF-I PC fraction) contained all of the TIF needed for faithful initiation in the Acanthamoeba cell-free system. This TIF extract was quick frozen and stored in liquid nitrogen. Specific initiation of transcription was assayed using a runoff assay as described previously (15). Protein concentrations were estimated by the method of Lowry et al. (16) and RNA polymerase activities on calf thymus DNA estimated by the DE-81 paper disc assay as previously described (17), except nucleotide and salt (KCl) concentrations were as in the in vitro runoff assay. Alpha-amanitin was used to distinguish RNA synthesis due to polymerase I, II, or III (11).

RESULTS

Ribosomal RNA transcription is terminated during the early stages of Acanthamoeba encystment

When starved for essential nutrients, Acanthamoeba castellanii undergoes a series of biochemical and morphological changes resulting in the formation of a dormant cyst (reviewed in 18). Stevens and Pachler (19) demonstrated that during the first few hours of experimentally induced

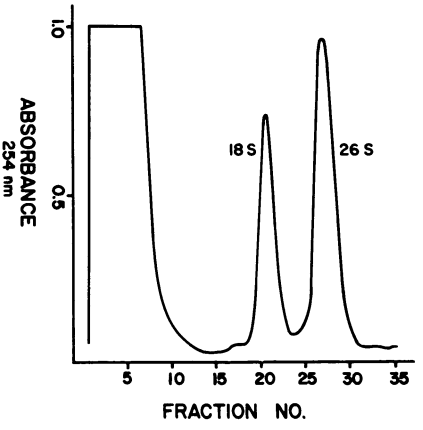


Figure 1. Sucrose gradient separation of Acanthamoeba RNA. RNAs extracted from A. castellanii were separated by isokinetic sucrose gradient rate zonal sedimentation as described in METHODS.

encystment, the rate of incorporation of [^{3}H]-uridine into rRNA was drastically reduced. They showed that this was due to a cessation of transcription and not to a change in uptake or further metabolism of [^{3}H]-uridine nor to a change in nucleotide pool sizes within the cell. Since our method of inducing encystment is different from their method, we wanted to verify that the time course of shutdown of transcription was the same under our conditions of cellular differentiation.

Labelling experiments were carried out on Acanthamoeba cells which were undergoing starvation-induced encystment in order to assess the temporal shutdown of rRNA synthesis. A typical absorbance profile of Acanthamoeba RNAs separated on an isokinetic sucrose gradient is shown in figure 1. The large peak in fractions 1-9 is due to absorbance of small RNAs (5S, tRNA, etc.) and of aurin tricarboxylic acid, a potent inhibitor of ribonucleases (20). Since the yield of total RNA extracted from different batches of cells differs slightly, the area under the 18S and 26S peaks was used to normalize the isotope incorporation data shown in figure 2. At each time point, pulse labelling with [^{3}H]-uridine was carried out for a total of 1 hr, 0.5 hr before thru 0.5 hr after the times shown in the figure. Curves for RNA pulsed at 2, 4, 6, and 8 hr time points are shown; data for pulse labellings at 10 and 12 hr are omitted to keep the figure uncluttered. Immediately following induction of encystment, the rate of synthesis of rRNA begins to decrease. By 8 hr postinduction, the transcription of rRNA has almost

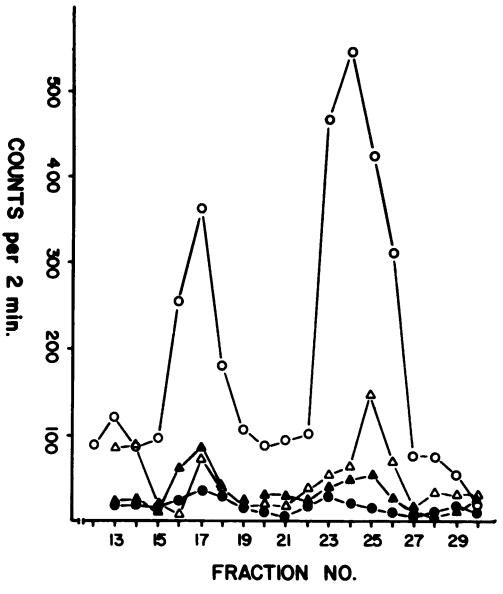


Figure 2. Pulse labelling of high molecular weight RNA from Acanthamoeba cells starved for various periods of time. Cells were transferred to Neff's encystment medium and pulse labelled with [^{3}H]-uridine for 1 hr as described in METHODS. RNA was extracted and separated by rate zonal sedimentation, the gradients fractionated and aliquots counted in a Triton-toluene based scintillant. Pulse times were (○) 2 hr, (△) 4 hr, (▲) 6 hr and (●) 8 hr.

completely ceased. At later time points, incorporation of label into rRNA is undetectable. This finding is in complete agreement with the more complete study done by Stevens and Pachler and demonstrates that the temporal events of cellular differentiation are unaltered by the method of induction used.

In vitro initiation in a cell-free system

A complete rRNA repeat unit from Acanthamoeba has been cloned and characterized (21). The start site for the transcription unit has been identified (22) and a subclone containing a 915 base pair DNA fragment capable of promoting correct transcription constructed (Fig. 3). We have shown (15) that when this DNA fragment, designated pAr4/Hha I (-420 to +495; +1 codes for the first base of the transcript, minus numbers precede the transcribed sequence), is correctly transcribed in vitro, a "runoff" RNA of 495 bases in length is produced due to termination at the end (+495) of the truncated DNA template (e.g. Figs. 3 and 4). Faithful transcription in this

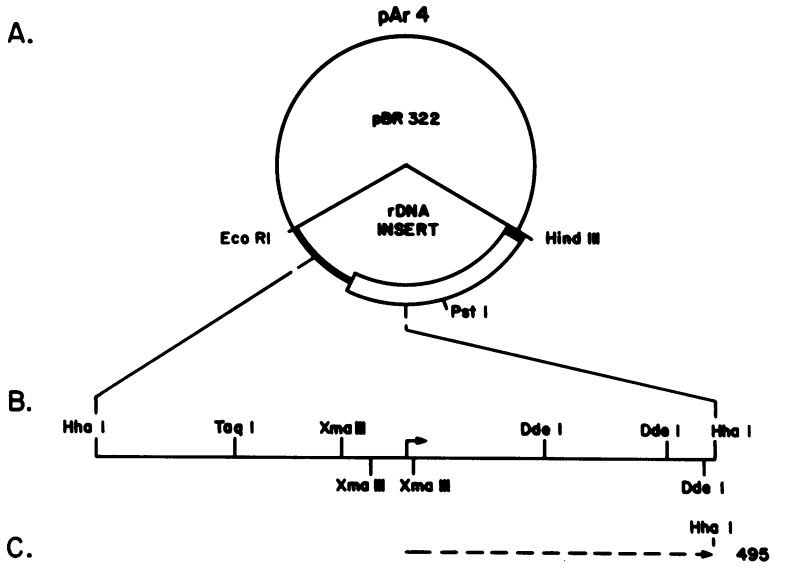


Figure 3. Map of pAr4/Hha I DNA fragment. (A) A map of pAr4 showing the pBR322 plasmid (light line), the nontranscribed spacer region (heavy line), the external transcribed spacer (open box) and the 3'-end of the 18S coding region (filled box). (B) A map of the pAr4/Hha I DNA fragment used as template in in vitro transcription. The position of the transcription start site is indicated by the bent arrow. (C) The runoff transcript produced by in vitro transcription of pAr4/Hha I DNA.

system requires, in addition to the DNA template and RNA polymerase, a crude cellular extract (S100) containing one or more transcription initiation factors (TIF-I). In the absence of this component, purified RNA polymerase I is incapable of initiation at the correct start site for this transcription unit (cf. Fig 4, lane 1 and lane 2).

Levels of TIF-I are constant during experimentally induced encystment

Extracts of cells harvested at various times following induction of encystment were tested for their level of TIF-I by estimating the ability of the extracts to direct faithful initiation of rRNA transcription in the in vitro system. Extracts were assayed for protein content and RNA polymerase I activity, the latter by using calf thymus DNA as template (see INTRODUCTION). All of the S100 extracts had nearly the same protein and polymerase I content. This is in agreement with our earlier finding that the level of RNA polymerase I in starved cells remains constant through at least the first 16 hr of encystment (11). Nevertheless, for the in vitro

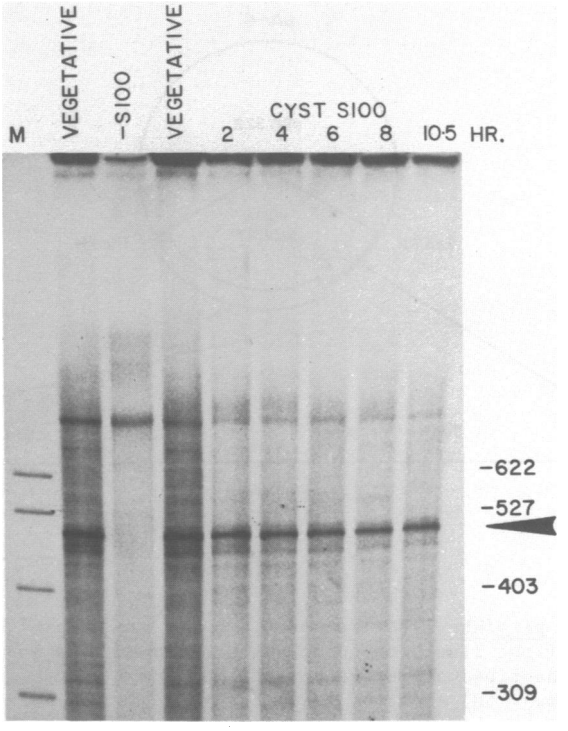


Figure 4. Levels of TIF-I in extracts prepared from cells starved for various periods of time. S100 extracts of cells incubated in Neff's encystment medium for various periods of time were prepared by the method of Weil et al. Extracts were tested for TIF-I level by assaying transcription in vitro in the presence of excess RNA polymerase I (30 munits/assay) purified from vegetative Acanthamoeba cells. The S100s contributed 5.6 munits/assay of polymerase I before supplementation. The 495 nucleotide runoff RNA is indicated by the arrowhead. The lane marked M contains pBR322/Msp I markers whose lengths are listed on the right side of the figure. Lanes marked VEGETATIVE contained S100 from cells growing in log phase. The -S100 lane had no S100 added. Lanes marked Cyst S100 contained S100 prepared from cells starved for the number of hours indicated above each lane, 2 through 10.5 hr. The band at 915 nucleotides is an end-to-end transcript of the pAr4/Hha I DNA.

transcription assays, the amount of total protein added from each extract was carefully equalized. This also resulted in an equalization of the endogenous RNA polymerase I activity. Then a 5.5 fold excess (relative to the endogenous polymerase I activity) of highly purified (17), homologous RNA polymerase I was added. Under these conditions the limiting factor for specific initiation of transcription is the TIF-I, not the availability of polymerase. This was verified by titration of the extract with purified RNA

polymerase I (data not shown). The results of these assays are shown in figure 4. Extracts made from trophozoites (log phase cells) or cells which were 2, 4, 6, 8 or 10.5 hr into the encystment process synthesized identical amounts of the 495 base runoff RNA. This demonstrates that the amount of active TIF-I in all of these extracts is identical. Thus we conclude that since the in vivo transcriptional activity of the rRNA genes in these cells is drastically different (cf. Figs 2 and 4) while the TIF-I level is constant, the transcription of rRNA in these cells cannot be regulated by alteration in the level of the transcription factor required for specific initiation by RNA polymerase I.

During encystment, S100 extracts unsupplemented with RNA polymerase I lose the ability to initiate transcription

We showed previously that the number of RNA polymerase I molecules in starved Acanthamoeba cells remains constant during the first 16 hr of encystment (11). This conclusion was based upon measurement of the rate of transcription of calf thymus DNA in the presence of high levels of α-amanitin. This assay is a measure of the total catalytic potential of the polymerase I in an extract without interference from events involved in specific initiation at the promotor of the rRNA transcription unit because the highly damaged template provides a large number of artificial initiation sites (nicks, single - stranded gaps and ends with single - stranded extensions). However, we were interested in testing whether RNA polymerase I from starved cells was as capable of specific initiation at the rRNA promotor as polymerase from log phase cells. To determine this, the S100 extracts prepared from cells which had been starved for various periods of time were assayed in the same manner as above except no purified RNA polymerase I was added to supplement the extracts. The amounts of extract used were adjusted slightly so that equal units of RNA polymerase I (based on transcription of calf thymus DNA) were present in each reaction (see above). The results are shown in figure 5. Significantly, even though they have equal numbers of RNA polymerase I molecules active in transcription of damaged DNA templates, extracts made from cells which had been starved lose their ability to specifically initiate transcription at the promotor.

The loss of the specific 495 base runoff RNA observed in the starved - extract assays of figure 5 could occur because of an increase in the amount or activity of RNase in the extracts or due to the presence of an inhibitor in the extracts from starved cells. These possibilities were ruled out by assaying mixtures of 10.5 hr and trophozoite S100 extracts (data not

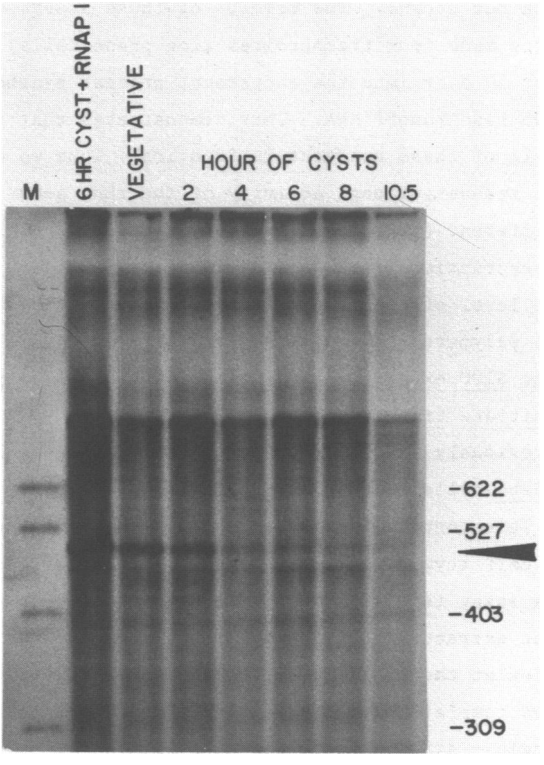


Figure 5. Levels of faithful initiation-competent RNA polymerase I in extracts prepared from cells starved for various periods of time. S100 extracts from the experiment depicted in figure 4 were assayed for in vitro transcription without supplementing the polymerase endogenous to the extracts with purified RNA polymerase I. Autoradiography of this polyacrylamide gel was carried out much longer than that in figure 4 due to the lower level of transcription occuring in the unsupplemented extracts, thus increased background is apparent. The arrowhead indicates the 495 runoff RNA product. Lane M is pBR322/Msp I markers. 6HR CYST + RNAP I is a lane containing the product resulting from supplementation of the extract prepared from cells starved for 6 hr with purified RNA polymerase I from vegetative cells. The lane marked VEGETATIVE contained S100 made from log phase cells. The lanes marked HOUR OF CYSTS contained S100s made from cells starved for the number of hours indicated, 2 through 10.5 hr. The band at 915 in this autoradiogram is an end-to-end transcript of the pAr4/Hha I and serves as an internal control for nonspecific transcription. The 10.5 hr lane is unusually light in this particular gel (compare with the identical S100 in figure 4).

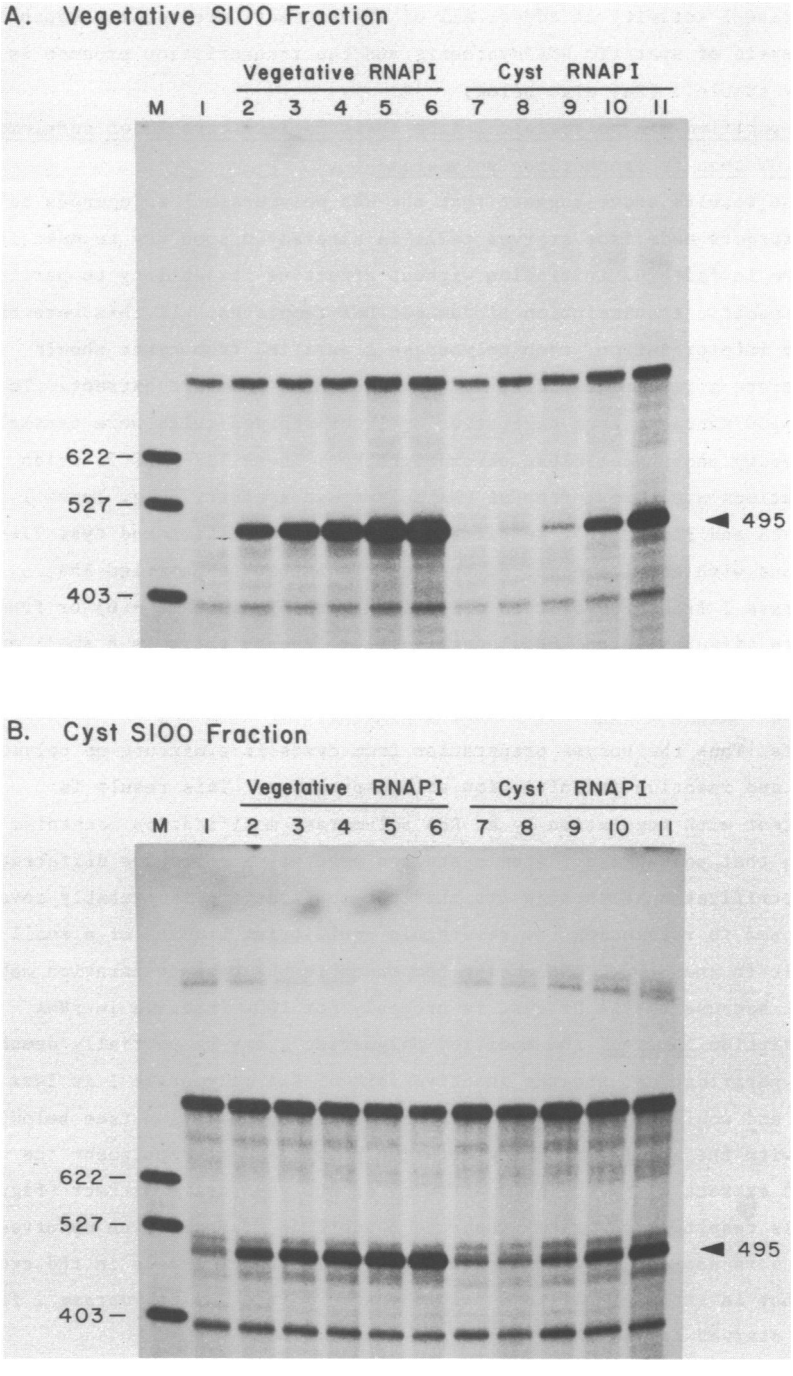
shown). Further, the results of figure 4 demonstrate that the only difference between the trophozoite and cyst extracts resides in the RNA polymerase used to complement the extracts; when sufficient trophozoite RNA

polymerase I activity is added, all of the extracts are equally capable of high levels of specific RNA synthesis and the transcription product is equally stable. (See also below)

Highly purified RNA polymerase I from cysts is less capable of supplementing cyst S100 than is trophozoite polymerase

The results above suggest that the RNA polymerase I endogenous to the S100 extracts made from starved cells is altered in some way to make it inactive in faithful initiation without affecting its ability to participate in nonspecific transcription of damaged DNA templates. If this were the correct interpretation, then polymerase I purified from cysts should demonstrate a reduced capacity to complement the inactive extract. To test this, S100 extracts from vegetative or 11 hr starved cells were partially purified by phosphocellulose chromatography. These TIF-I PC fraction preparations are almost free of RNA polymerase activity (e.g. lanes 1 in Figure 6A and Figure 6B). We titrated both the vegetative and cyst TIF-I PC fractions with equal amounts of the heparin-Sepharose-purified RNA polymerase I from trophozoites (figures 6A and 6B, lanes 2 - 6) or from 11 hr cysts (figures 6A and 6B, lanes 7 - 11). Though there is a small amount of stimulation by the purified cyst RNA polymerase I, it is 3 - 5 fold less efficient than the vegetative cell enzyme at complementing the deficient extracts. Thus the enzyme preparation from cysts is a mixture of polymerase active and inactive in initiation at the promoter. This result is consistent with regulation by an RNA polymerase modification mechanism. The finding that polymerase I from cysts and vegetative cells are different even after purification also suggests that the modification is probably covalent, as opposed to reflecting the reversible equilibrium binding of a small molecule in the S100. The active component in the cyst preparation may be present because the 11 hr cyst is probably not 100% inactive in rRNA transcription in vivo, the modified polymerase I may be partially demodified during purification, and the inactive form of RNA polymerase I is less stable and would be preferentially lost during purification (see below). Along with the ability of the vegetative polymerase to supplement the starved extract up to the same activity as the vegetative extract (figure 4), this result demonstrates that the shutoff in transcription observed in figure 5 is not due to a change in RNase or inhibitor levels in the crude S100, but is attributable to a stable change in the RNA polymerase I found in the starved cells.

In the experiment of Figure 4, no effort was made to use exactly equal


A. Vegetative SIOO Fraction
Vegetative RNAPI
Cyst RNAPI
M I 2 3 4 5 6 7 8 9 10 11
622 —
527 —
403 —
◄ 495
B. Cyst SIOO Fraction
Vegetative RNAPI
Cyst RNAPI
M I 2 3 4 5 6 7 8 9 10 11
622 —
527 —
403 —
◄ 495

amounts of TIF-I PC fraction in parts A and B, so differences between levels of transcription in Figure 6A and 6B are incidental. We do note that there is about a 2 fold difference between the transcriptional activity of the cyst and vegetative TIF-I PC fractions. This difference must be attributed to differences in the yield of TIF through the phosphocellulose purification step since the activity of TIF in the S100s from the two cell types is identical (Figure 4). Because of this difference, we cannot unequivocally rule out a role in regulation of altered TIF levels, stability, or association with other cellular components. However, because the direct measurements of unpurified extracts (Figure 4) show no difference in TIF activity and the difference in the purified fraction is only 2 fold, we think it unlikely that this is the major control mechanism.

Highly purified RNA polymerase I from cysts is more heat labile than is trophozoite polymerase

We have shown that the general catalytic properties of polymerase I do not change during the encystment process (23). These properties include divalent metal ion preference, ability to transcribe double- or single-stranded DNA templates, ionic strength optimum, α-amanitin sensitivity and specific activity on damaged DNA templates. However, we expected that some property of the polymerase should be altered by modification besides its ability to initiate at promoters. Thus, we compared the rate of heat inactivation of polymerase I purified from

Figure 6. Titration of partially purified S100 extracts with RNA polymerase I purified either from vegetative or starved cells. S100 extracts prepared from vegetative and 11 hr starved cells were fractionated by phosphocellulose chromatography, as described in METHODS, and the TIF-I PC fractions substituted for crude S100 in the in vitro transcription assay. The assay mixtures were supplemented with increasing amounts of RNA polymerase I purified from vegetative cells (lanes marked Vegetative RNAP I) or from cells starved for 11 hr (lanes marked Cyst RNAP I). (A) In vitro transcription utilizing the TIF-I PC fraction from vegetative cells supplemented with the following milliunits of purified vegetative or cyst RNA polymerase I: 0 munits (lane 1), 2.08 munits (lanes 2 and 7), 3.47 munits (lanes 3 and 8), 5.2 munits (lanes 4 and 9), 10.4 munits (lanes 5 and 10), and 20.8 munits (lanes 6 and 11). (B) In vitro transcription utilizing the TIF-I PC fraction prepared from 11 hr starved cells. In each lane RNA polymerase I was supplemented as in (A). Each of the assays in (A) and (B) also contained 0.48 munits of polymerase I [determined by incorporation of [^{3}H]-UMP into RNA using calf thymus DNA as template (17)] and 4.4 micrograms of protein from the vegetative TIF-I PC fraction or 1.52 munits of polymerase I and 5.6 micrograms of protein from the cyst TIF-I PC fraction. The arrowheads mark the positions of the runoff RNA. Lane M represents markers from Msp I digest of pBR322. The band at 915 nucleotides is an end-to-end transcript of the pAr4/Hha I DNA.

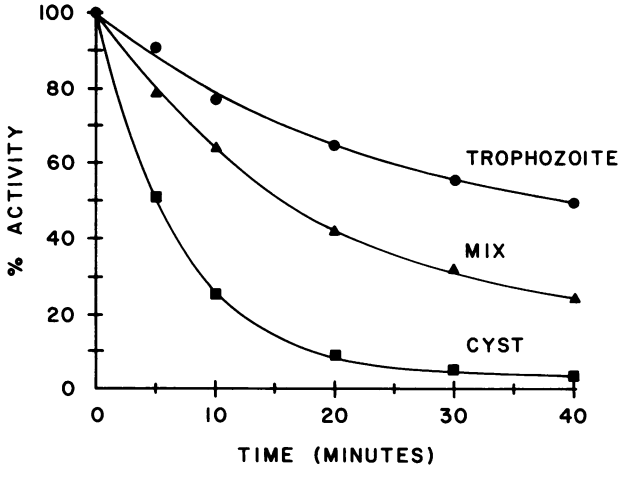


Figure 7. Heat inactivation of RNA polymerase I purified from vegetative and starved cells assayed on calf thymus DNA. 8.9 units/ml of RNA polymerase I purified from vegetative cells (●), 7.6 units/ml of enzyme purified from 11 h starved cells (■), and 8.6 units/ml of an equal mix of RNA polymerase I from vegetative and starved cells (▲) were incubated in storage buffer at 44°C for the times indicated, duplicate aliquots removed and chilled on ice. RNA polymerase activity was determined by estimating the incorporation of [^{3}H]-UMP into RNA using calf thymus DNA as template (17).

vegetative and starved cells. Purified enzymes were diluted in storage buffer to equal concentrations and heated to 44°C for various periods of time. Aliquots of the heated enzyme were assayed for incorporation of [^{3}H]- uridine into RNA using commercial calf thymus DNA as a template (17). The data of figure 7 show that the polymerase isolated from starved cells denatures more rapidly than the enzyme from trophozoites. The average time (3 experiments) required to 50% inactivate polymerase from vegetative cells is 5 times that required to inactivate cyst polymerase to the same extent. Heating of a mixture of the enzymes gave an intermediate profile expected from the addition of two independent zero-order reactions. This result demonstrates that the differences observed are inherent in the polymerase molecules and are not a reflection of additional stabilizing or destabilizing components in the preparations.

Any specific initiation activity observed in the cyst polymerase preparation is presumably due to unmodified enzyme. This unmodified enzyme should be identical to the vegetative polymerase and should, therefore, have the same heat inactivation properties as the vegetative enzyme. This notion

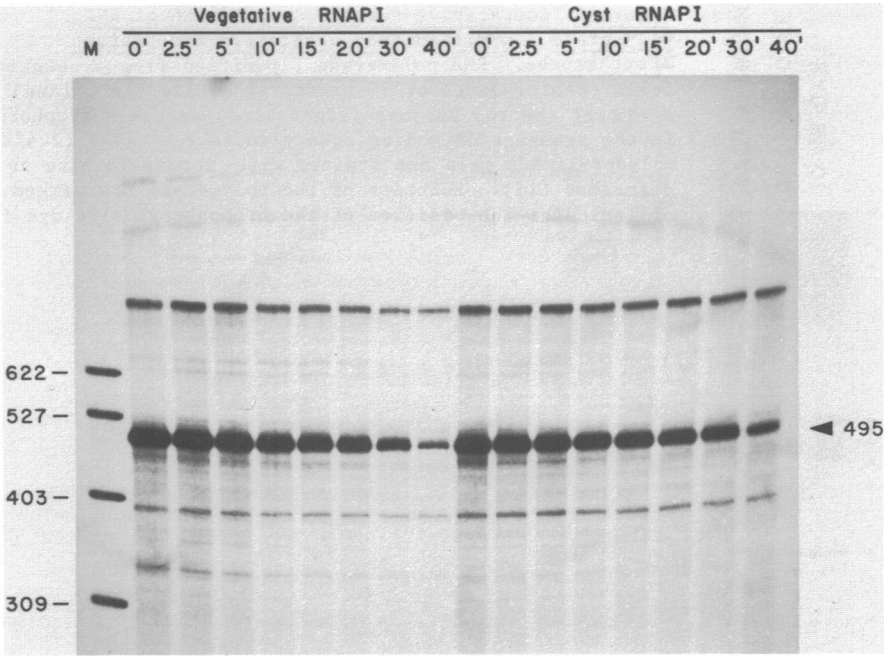


Figure 8. Heat inactivation of RNA polymerase I from vegetative and starved cells assayed in the specific runoff assay. RNA polymerase I purified from vegetative cells (lanes 1-8) or 11 hr starved cells (lanes 9-16) was incubated in storage buffer at 44°C for the indicated times, aliquots removed and used to initiate transcription in vitro. Total activity of RNA polymerase I from starved cells initially added to the assays was approximately three times that of the vegetative RNA polymerase I added (20 munits), as determined by the [^{3}H]-UMP calf thymus DNA assay (17). This was done to equalize the amount of specific transcription observed at the first time points (lanes 1 and 9). Lane M represents markers from Msp I digest of pBR322.

can be tested by comparing heat inactivation profiles using the specific runoff assay. Purified enzymes were heated as above and aliquots were utilized in the runoff assay as described in the legend to figure 8. A three fold greater amount of the cyst enzyme was added to the runoff assays so that initially (time zero) the specific initiation activity in the two sets of assays would be nearly identical. It is clear that the heat inactivation profile of the active enzyme in the vegetative and cyst preparations is identical (Figure 8).

The gross subunit architectures of RNA polymerase I from trophozoites and cysts are identical

In an effort to determine the nature of the modification of the RNA

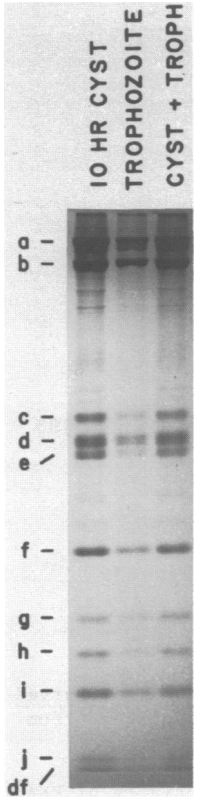


Figure 9. Comparative subunit composition of RNA polymerase I purified from vegetative and starved Acanthamoeba. RNA polymerase I purified from vegetative cells (center lane) or 10 hr starved cells (left lane) or a mix of the two enzymes (right lane) were electrophoresed in the presence of sodium dodecylsulfate in 12%T (2.4%C) polyacrylamide gels and stained with coomassie blue as described (17). Subunits of the polymerase are marked a-j. df indicates the position of the bromophenyl blue dye front.

polymerase, we compared the subunit composition of polymerase I purified from trophozoites and 10 hr cysts (figure 9). Polypeptides marked a - j have previously been shown to be subunits of the Acanthamoeba RNA polymerase I (17, reviewed in 2). The same set of subunits is found in both trophozoite and cyst polymerases and no new subunits appear in stoichiometric amounts. Therefore, the modification of the polymerase is not a loss or gain of a subunit, but rather must be some other type of modification (see Discussion).

DISCUSSION

Recently, methods have been developed which allow the transcription of rRNA genes from deproteinized recombinant DNA templates in cell-free systems (10, 24-28; 15). Studies with these in vitro systems have revealed that transcription of eukaryotic rRNA requires a protein component or components in addition to RNA polymerase I. In apparent analogy to transcription initiation factors associated with RNA polymerase II and III mediated

transcription systems, TIF-I appears to act by formation of a stable complex with the DNA template (29,30, C. Iida, unpublished). Since separate TIFs are required by each of the transcriptional systems (4,31) the TIF itself becomes a potential target for regulatory mechanisms affecting expression of the different gene types transcribed by the distinct RNA polymerase classes. This is especially true for RNA polymerase I mediated transcription of rRNA since this is the sole transcription unit recognized by this polymerase and TIF. Indeed, Grummt (10) found that the assayable levels of TIF-I varied between rapidly and slowly growing Ehrlich ascites tumor cells. Since the rate of transcription of rRNA varies in direct proportion to the growth rate of these cells, she has suggested that the intracellular level of TIF-I may be modulated in order to regulate the transcriptional activity of rRNA genes in mouse cells.

The rate of transcription of rRNA is similarly regulated in response to growth rate in the small free-living amoeba, Acanthamoeba castellanii. When starved, this organism ceases growth and cell division and differentiates into a dormant cyst (18). The rate of synthesis of rRNA rapidly declines following experimental induction of encystment, becomming undetectable by 8-12 hr postinduction (19) [figures 2 and 5]. In contrast to the findings in the mouse study, we show here that the level of TIF-I in cellular extracts remains unaltered up to 10.5 hr postinduction (figure 4). Therefore we conclude that it is not a decline in the number of TIF-I molecules in the cell that regulates the transcriptional activity of rRNA genes in Acanthamoeba.

In the Acanthamoeba system, we present evidence that RNA polymerase modification is responsible for regulation of rRNA transcription. Extracts made from rapidly growing (vegetative) and from starved cells are equally capable of rapid rRNA transcription when supplemented with an excess of RNA polymerase I purified from vegetative cells (figure 4). Extracts made from vegetative cells contain endogenous RNA polymerase I which obviates the need for supplementation by purified enzyme (figure 5). In contrast, extracts made from starved cells are incapable of specific transcription unless supplemented with purified RNA polymerase I (figure 5). The two extracts are equal in polymerase I activity, however, when assayed on damaged DNA. This assay is thought to measure the number of RNA polymerase molecules capable of chain elongation after initiation at nonspecific template sites resulting from DNA damage. This finding is in agreement with our earlier demonstration that the number of RNA polymerase molecules remains constant

in differentiating Acanthamoeba cells (11). Thus, the present study shows that only the ability to faithfully initiate on the rRNA promoter is lost by the polymerase from starved cells, not the general catalytic activity of the enzyme.

The modification of polymerase I leading to its loss of ability to faithfully initiate RNA chains at the promotor is a stable alteration of its structure, but not a change in its subunit architecture. Homogeneous RNA polymerase I purified from cells which have been starved is significantly less able to complement the inactive extracts than is polymerase purified from vegetatively growing cells (figure 6). In addition, the purified polymerase from starved cells is heat inactivated 5 times faster than the enzyme from trophozoites (figure 7). This difference is observable in nonspecific assays, using calf thymus DNA, but not in specific runoff assays involving faithful initiation at the promoter since only the unmodified enzyme is active in the latter (figure 8). Since the gross subunit compositions of RNA polymerase I from starved and growing cells are identical (figure 9), we believe that the difference between the trophozoite and cyst enzyme must arise from a phosphorylation, adenylylation or similar modification. Further, the modification must result in a subtle alteration of the catalytic capabilities of the enzyme. Modification is not detected by assaying nonspecific transcription of damaged calf thymus DNA (enzyme purified from cysts and trophozoites has the same specific activity, unpublished) nor by comparing the catalytic properties (ionic strength optimum, divalent metal ion preference, single- or double-stranded DNA preference) of the enzyme from growing and starved cells (23). Since the initial observation of *in vitro* phosphorylation of a eukaryotic nuclear RNA polymerase made by one of us (MRP) in 1973 (32), the literature has been replete with reports of phosphorylation of these enzymes (eg. see the review in 33). None of these reports have shown any direct regulatory link between the modification reaction and specific gene expression. This lack of a demonstrable effect on transcription may be due to the use of nonspecific assays. Faithful initiation such as in the study presented here may be necessary to demonstrate an effect. Structural comparisons of the polymerases purified from growing and starved cells are currently underway to try to determine the nature of the putative modification.

In collaboration with Grummt (34), one of us (MRP) has shown that transcription of rRNA genes *in vitro* is species specific; DNA templates from one organism cannot be optimally transcribed in S100 extracts from another

(see also 25). Muramatsu and coworkers (35) have shown that the species specificity for a human-mouse pair resides in a non RNA polymerase I containing part of the cellular extract fractionated on phosphocellulose. In light of this species specificity, it is intriguing that it appears there may be several distinct mechanisms for regulating rRNA transcription in different eukaryotic cell types. In mouse, Grummt (10) has reported preliminary evidence that gene activity is regulated by modulation of TIF-I levels in the cell. Tjian and coworkers (36) have presented convincing in vitro studies that the protein product of the early gene A of Simian Virus 40, the large-T antigen, directly stimulates in vitro transcription of human rRNA genes, but not of other genes. We present evidence here that modification of the RNA polymerase I leads to regulation of the rRNA transcription unit in a differentiating protist. The latter is the first demonstration of regulation of eukaryotic gene expression by polymerase modification.

One of the predictions of our model is that as rRNA transcription is shut down, there should be a gradual decrease in the density of RNP fibrils observed in Miller spreads of genomic rRNA repeat units. (This prediction assumes that there is no cooperativity in RNA polymerase I binding and initiation.) In amphibians, this gradual decrease has not been reported and instead, each rRNA repeat unit appears to be either completely "on" or completely inactive (reviewed in 37). However, this all-or-none transcription is not a general phenomenon since there are various levels of rRNA transcriptional activity observed in Miller spreads of the silk moth, Bombyx mori (38) and of yeast (39). A systematic electron microscopic study of this difference in apparent regulatory mechanisms has not been carried out (B. Hamkalo, personal communication). We are currently testing Acanthamoeba by Miller spreading to determine how the nascent fibril density of the transcription units change during encystment.

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