Coordinate Regulation of Ribosomal Component Synthesis in *Acanthamoeba castellanii*: 5S RNA Transcription Is Down Regulated during Encystment by Alteration of TFIIIA Activity

JENNIFER L. MATTHEWS, MICHAEL G. ZWICK, AND MARVIN R. PAULE*

Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

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Transcription of large rRNA precursor and 5S RNA were examined during encystment of *Acanthamoeba castellanii*. Both transcription units are down regulated almost coordinately during this process, though 5S RNA transcription is not as completely shut down as rRNA transcription. The protein components necessary for transcription of 5S RNA and rRNA were determined, and fractions containing transcription factors comparable to TFIIIA, TFIIIB, and TFIIIC, as well as RNA polymerase III and a 3′-end processing activity, were identified. Regulation of 5S RNA transcription could be recapitulated in vitro, and the activities of the required components were compared. In contrast to regulation of precursor rRNA, there is no apparent change during encystment in the activity of the polymerase dedicated to 5S RNA expression. Similarly, the transcriptional and promoter-binding activities of TFIIIC are not altered in parallel with 5S RNA regulation. TFIIIB transcriptional activity is unaltered in encysting cells. In contrast, both the transcriptional and DNA-binding activities of TFIIIA are strongly reduced in nuclear extracts from transcriptionally inactive cells. These results were analyzed in terms of mechanisms for coordinate regulation of rRNA and 5S RNA expression.

The number of ribosomes in the cell is regulated to correspond to the quantitative requirement for protein synthesis. Therefore, the rate of biosynthesis of ribosomes varies with the cellular proliferation rate, in response to cellular growth without cell division (e.g., cardiac hypertrophy [1]), and with significant changes in protein synthesis rates in secretory tissues (e.g., the *Drosophila* paragonal gland [29] [reviewed in reference 22]). The synthesis of the 80 to 90 components of the ribosome must be coordinately up or down regulated to correspond to changes in ribosome elaboration rate. The mechanisms leading to balanced synthesis of these components is poorly understood. For the ribosomal proteins, regulation occurs at several steps in the biosynthetic pathway, including transcription by RNA polymerase II, processing, translation efficiency, and mRNA stability. The critical regulated step depends on the particular ribosomal protein and the species (2, 3, 19–21, 30). rRNA and 5S RNA are transcribed by RNA polymerases I and III, respectively. Thus, various ribosomal components are transcribed by each of the three eukaryotic RNA polymerases. Expression of the stable RNAs is regulated at the transcriptional level. Therefore, the problem of coordinating their expression is, at least in part, one of coordinating transcription of their genes by distinct transcriptional systems. This study investigates the mechanisms involved in coordinating precursor rRNA and 5S RNA transcription in *Acanthamoeba castellanii*.

When starved for essential nutrients, *A. castellanii* leaves the growth/division cycle and undergoes cellular differentiation into a dormant cyst. When this occurs, rRNA transcription, which in the proliferating cell is 75% of pulse-labeled RNA, ceases completely (Fig. 1) (23, 27). We show here that 5S RNA transcription is coordinately modulated with rRNA. Transcription of rRNA is regulated by modification of the enzyme responsible for its synthesis, RNA polymerase I. Polymersese I purified from transcriptionally active cells is active both in nonspecific assays (transcription of damaged templates) and in specific, promoter-dependent assays in vitro. However, polymerase purified to apparent homogeneity from cysts cannot initiate from promoters even though it retains full activity in the nonspecific assay (4, 23). Structurally, a change in the electrophoretic mobility of the 39-kDa subunit correlates with this regulation. This subunit is the eukaryotic homolog of the bacterial α subunit and is one of the subunits which is common to RNA polymerases I and III (8). Therefore, it was appealing to suggest that regulatory modification of this common subunit might coordinate the activity of the two transcriptional systems, though in the case of polymerase III, a number of genes would be affected. Alternatively, 5S RNA transcription could be specifically targeted for regulation by alteration of the activity of one of the transcription factors required for promoter recognition, and coordination of expression of the multiple genes would be accomplished by a more indirect mechanism.

5S RNA transcription has been shown to be regulated by alteration of each of the transcription factors involved in its expression. In *Xenopus* oogenesis and embryonic development, the 5S RNA gene-specific transcription factor TFIIIA has been implicated. Though precisely the mechanism involved in the differential regulation of the oocyte-type and the somatic-type 5S RNA gene copies is complicated by the involvement of chromatin, times of replication of the two gene types, and differences in the rates of formation of transcription complexes on the genes, it is the level of TFIIIA in the nucleus which fundamentally affects 5S RNA expression rates (reviewed in references 31 and 33). During mitosis, repression of 5S RNA transcription involves phosphorylation of a different transcription factor, TFIIIB (15). Similarly, treatment of *Drosophila* Schneider S2 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor-promoting phorbol ester, results in stimulation of both rRNA and 5S RNA transcription without affecting polymerase III activity levels, suggesting one of the general transcription factors as the target (13). TFIIIB was subsequently shown to be increased in amount in TPA-treated cells, on the basis of activity and immunological cross-reactivity with...
a subunit-specific (TATA-binding protein antibod (14). TFIIIB activity was found to rapidly decrease in amount fol- owing treatment of mammalian cells with cycloheximide, sug- gesting that it turns over more rapidly than the other compo- nents (28). TFIIIC is also a target for regulation, especially during viral infection or virus-mediated transformation (re- viewed in reference 31). Thus, one can identify cases in which each of the three transcription factors required for 5S RNA transcription are regulated.

The data presented here for Acanthamoeba development uphold the notion that a transcription factor rather than poly- merase III is the target of regulation. The activity or level of TFIIIA, the 5S RNA-specific transcription factor, is drastically decreased in cyst extracts, in parallel with the shutdown of 5S RNA transcription. The level of the other general polymerase III transcription factors and RNA polymerase III are un- changed until later in the development process. These results are discussed in terms of mechanisms for coordinating trans- cription of rRNAs.

**MATERIALS AND METHODS**

**Cultivation of cells.** A. castellanii trophozoite cells were grown to a density of 2 × 10^5 to 4 × 10^6/ml and harvested as described previously (10, 24). Encysting cells were derived from vegetative cells by treatment with 0.1 M flowthrough from a phosphocellulose column (phosphocellulose 0.1 M fraction) which con- tained the 3H and 32P in a Beckman LS7800 scintillation counter. The 3H counts per minute were corrected accordingly.

**DNA purification.** DNA from Acanthamoeba.* 126 to 280 returned to near baseline. The supernatant was collected by centrifugation in an Eppendorf centrifuge (5 min at maximal speed) and resuspended in 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 to 3% glycerol, 2 mM DTT, 1 μg of a-tamanitin per ml, 5 μg/ml of a-tubulin (Sigma), 0.5% N-lauroyl sarcosine, 25 mM sodium citrate (pH 7.0), 100 mM [3H-mercaptoethanol], and 100 mM MGG (5 mg/ml) of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 54.5 mM of 7.5 M ammonium acetate, and 410 μl of absolute ethanol. The RNA was collected by centrifugation in an Eppendorf concentrator for 30 min at maximal speed. The pellets were washed with 400 μl of 70% (vol/vol) ethanol and briefly dried under vacuum. The RNA products were suspended in 6 μl of polyacryl- amide gel electrophoresis loading buffer (90% deionized formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol) and analyzed by a 6% denaturing polyacrylamide gel. Radiative RNA was visualized and quantified with a Molecular Dynamics PhosphorImager and ImageQuant 3.2 software.

**TFIIIA gel shift assay.** The 234-bp Xba-I-EcoRI fragment of pAC5s3dIII-34 (34 containing Acanthamoeba 5S RNA from −33 to +178 was labeled by a fill-in reaction with T4 DNA ligase (5 μg) and 32P-labeled [α-32P]dATP (3,000 Ci/mmol), 100 μM each of ATP, GTP, and CTP, and 100 μM each of 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT) were incubated at 25°C for 20 min. The reaction products were processed as described above.

**Preparation of nuclear extracts.** Nuclear extracts from vegetative and encysted A. castellanii cells were prepared as described previously (24).

**Fractionation of A. castellanii trophozoite and cyst nuclear extracts.** The buffer HEG₃ contains 50 mM N-2-hydroxyethylamino-N-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, and 20% (vol/vol) glycerol. HEG₃ is HEG buffer with 10% (vol/vol) glycerol. Whatman P1 (phosphocellulose) was pre- pared according to the manufacturer’s directions and equilibrated with HEG₃ containing 100 mM KCl. All operations were performed at 0 to 4°C. Nuclear extract (approximately 100 mg of total protein) prepared from trophozoite cells or 10-h encysted cells was diluted with HEG₃ to 100 mM KCl and applied to a P1 column (1.4 cm × 15 cm). The elution buffer contained 0.3 M NaCl, 10 mg of protein per ml of bed volume) at a linear flow rate of 7.8 cm/h. Protein eluting from the column was monitored by A₄₅₀. The flowthrough was collected, and the column was extensively washed with HEG₃ containing 100 mM KCl. The bound protein was sequentially step eluted from the column with HEG₃ containing 0.3, 0.45, 0.65, and 1.5 M KCl. Protein was collected from each fraction until all of the A₄₅₀ absorption approached near baseline.

**RNA polymerase assays.** Nonspecific transcription assays were performed in the presence of various levels as previously described (24). In vitro transcription assays. Standard transcription assays were performed under the following conditions: 50 mM HEPES-KOH (pH 7.9), 90 mM KCl, 7.5 mM MgCl₂, 1 to 3% polyvinylpyrrolidone, 2 mM of a-aminanilin per ml, 600 μg/ml of a-tubulin (Sigma), 3% glycerol, 2 mM dNTP, 5% (vol/vol) of 100% phenol, and 40 ml of supercoiled plasmid DNA (pBS + S53, containing Acanthamoeba 5S DNA from −126 to +219 in pBS + [17]). To start the reactions, 3 μl of nuclear extract or 2 μl of the fractions indicated was added to the other premixed components to give a final volume of 30 μl. The reaction mixtures were incu- bated at 25°C for 75 min. The reactions were terminated by freezing on dry ice and adding 5 μl of proteinase K (10 mg/ml) and 6 μl of stop mix (250 mM NaCl, 1% SDS, 20 mM Tris-HCl [pH 7.6], 5 mM EDTA). The samples were then incubated at 25°C for 30 min. The reactions were precipitated by the addition of 300 μl of 100% ethanol and centrifuged for 30 min at maximal speed. The pellets were washed with 400 μl of 70% (vol/vol) ethanol and briefly dried under vacuum. The RNA products were suspended in 6 μl of polyacryl- amide gel electrophoresis loading buffer (90% deionized formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol) and analyzed on a 6% denaturing polyacrylamide gel. Radiative RNA was visualized and quantified with a Molecular Dynamics PhosphorImager and ImageQuant 3.2 software.

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The 172-bp tRNA fragment (50 to 100 ng) was labeled by a fill-in reaction performed with 20 μM each of [α-32P]dATP and [α-32P]dCTP and 100 μM each dGTP and dTTP as described above. After the 20-min incubation at 30°C, both dATP and dCTP were added to 100 μM and the incubation continued for an additional 10 min. The labeled fragment was purified with Qiaex (Qiagen).

The gel shift binding reaction mixtures contained 2 μg of double-stranded poly(dA-dT) and either trophozoite or cyst 0.65 M phosphocellulose fraction (6.3 μg of protein) or 1 μl of phosphocellulose-KCl gradient-purified TFIIIC in a final reaction volume of 20 μl. The buffer conditions for the assay were 90 mM KCl, 50 mM HEPES-KOH (pH 7.9), 7.5 mM MgCl2, 1 mM DTT, and 1 to 2% glycerol. After a 10-min incubation at 25°C, approximately 30,000 cpm of the labeled tRNA fragment (0.5 to 2 ng) was added and the reaction mixture was incubated for an additional 20 min. To each reaction mixture, 3 μl of 50% glycerol was added, and the entire reaction mixture was immediately loaded onto a non-denaturing 5% polyacrylamide gel. The gel and buffer conditions were as described above for the TFIIA EMSA, except that the gels were run for approximately 3 h.

The double-stranded oligonucleotides used to compete for TFIIIC binding were prepared as follows. Complementary oligonucleotides were synthesized by Macromolecular Resources, Colorado State University. Equal molaramounts of the oligonucleotides were mixed and annealed by standard methods. The annealed oligonucleotides had a four-nucleotide 5' overhang on both ends which was filled in with DNA polymerase I Klenow fragment and unlabeled deoxyribonucleotide triphosphates (as described above). The 24-bp B-box oligonucleotide contained the B-box consensus sequence (underlined): GATCCTAGGTTTCGAATCCTAGATC. The 24-bp nonspecific oligonucleotide contained a random sequence: GATCCAGCGGATCTAGAGATC.

RESULTS

Transcription of 5S RNA and rRNA is coordinately down regulated in encysting Acanthamoeba cells. Cultures were pulse-labeled to evaluate the rates of large precursor rRNA and 5S RNA transcription during encystment of A. castellanii. It has been demonstrated previously that there is no change in the uptake or processing of labeled uridine during experimentally induced encystment, and so labeling of RNAs in vivo is a measure of their transcription rates (27). When cells are transferred to starvation medium, they leave the growth/division cycle and begin differentiation into dormant cysts. In the early stages of this process, rRNA transcription is shut off (4, 23, 27) (Fig. 1). Similarly, 5S RNA expression is reduced in parallel with rRNA (Fig. 1). Unlike rRNA transcription, however, 5S RNA transcription does not decline to undetectable levels but retains approximately 10 to 15% of its maximal transcriptional activity seen in log-phase cells. This difference is consistently observed and was the first indication that the two transcription units utilized distinct mechanisms for regulation.

The mechanism responsible for the regulation of 5S RNA transcription in A. castellanii has been investigated by fractionation and characterization of the individual components of the RNA polymerase III transcription system. The phosphocellulose fractionation pattern of Acanthamoeba nuclear extracts is similar to those described in other systems. A trophozoite nuclear extract was fractionated on phosphocellulose into 0.1, 0.3, 0.45, 0.65, and 1.5 M KCl fractions. Utilizing a nonspecific transcription assay which exploits the different sensitivities of the three RNA polymerases to α-amanitin (12), RNA polymerase III was determined to be predominantly in the 0.45 M fraction. The remaining fractions were characterized in a reconstituted in vitro transcription assay (Fig. 2). The fractions were assayed alone and in all possible combinations for the ability to transcribe a homologous 5S RNA gene and heterologous tRNAiMet gene from...
Arabidopsis thaliana. A trophozoite nuclear extract assayed as a control (lanes 1 and 18) shows that both templates are accurately and efficiently transcribed. A greater amount of nonspecific transcription (high background) due to RNA polymerase III can be seen in all the lanes in which the 0.45 M fraction is included (for example, lanes 4, 9, and 11). No single fraction alone could transcribe either the 5S or tRNA gene (lanes 2 to 5). When two fractions were combined (lanes 6 to 11), only the combination of the 0.3 and 0.65 M fractions (lane 10) could support a very small amount of tRNA transcription (visible in the original phosphorimage). No combination of any two fractions gave detectable 5S RNA transcripts (lanes 6 to 11). Low levels of transcription from both templates can be seen in all of the various combinations of three fractions (lanes 12 to 15). Strong transcription is seen from the tRNA template with the combination of the 0.3, 0.45, and 0.65 M fractions (lane 15). However, only when four or more fractions (0.1, 0.3, 0.45, and 0.65 M) are combined is there substantial transcription from both the 5S and tRNA templates (lanes 16 and 17). The 1.5 M fraction is not required for transcription of either template but contains a 5S RNA 3′ processing activity that has been previously characterized (17). The processing of the 5S RNA transcripts can be seen with the addition of the 1.5 M fraction to the other four fractions (lanes 16 and 17).

This phosphocellulose fractionation pattern is similar to that of other systems. RNA polymerase III and the transcription factors are effectively, but not completely (as evident by the faint levels of transcripts seen in several lanes), separated from each other on a single phosphocellulose column. Only the fractions containing TFIIIB, RNA polymerase III, and TFIIIC (0.3, 0.45, and 0.65 M fractions, respectively) are required for transcription of the tRNA gene. The addition of the TFIIIA-containing 0.1 M KCl flowthrough fraction is required to reconstitute transcription of the 5S RNA gene.

Only the phosphocellulose 0.1 M fraction is altered in extracts prepared from encysted Acanthamoeba cells. A nuclear extract prepared from Acanthamoeba cells encysted for 10 h was fractionated on phosphocellulose into 0.1, 0.3, 0.45, 0.65, and 1.5 M KCl fractions. RNA polymerase III was determined to be predominantly in the 0.45 M fraction in a nonspecific polymerase assay. The remaining fractions were characterized according to the ability to substitute for the same fraction isolated from a trophozoite nuclear extract in a reconstituted in vitro transcription assay (Fig. 3). Extracts prepared from encysted cells typically show only about 12% of the transcriptional activity of the trophozoite extracts, which is consistent with in vivo pulse-labeling (Fig. 1). The fractionated extracts mimic these results (lane 1 versus lane 3). Again, the 1.5 M fraction is not required for transcription but contains a 5S RNA 3′ processing activity. The addition of the cyst 1.5 M fraction to either the cyst or trophozoite reconstituted system results in the production of a transcript of a single size (lane 2 or 4, respectively). No differences have been found in the activities of the 1.5 M fractions from trophozoite or encysted extracts. Thus, the processing activity does not appear to be regulatory (see also reference 17). When the trophozoite fractions were added individually to the cyst reconstituted system, only the trophozoite 0.1 M fraction could reconstitute activity of the cyst fractions to the trophozoite level (lanes 5 to 8). Thus, an activity in the trophozoite 0.1 M fraction could restore transcription to the cyst system.

The cyst fractions were tested for transcription-inhibitory activity by adding them individually to the trophozoite reconstituted system (lanes 9 to 12). There was essentially no change in the level of transcription seen with the addition of the cyst fractions. There was a slight increase in transcription seen with the addition of the cyst 0.45 M fraction (lane 11). This can be attributed to the addition of more RNA polymerase III (present in the cyst 0.45 M fraction) to the reaction mixture. These results establish that there is not a transcriptional inhibitor present in the cyst fractions.

To further determine if a particular cyst fraction was deficient in transcriptional activity, a single trophozoite fraction was substituted for the corresponding cyst fraction in the cyst reconstituted system (lanes 13 to 20). Again, only the trophozoite 0.1 M fraction could restore activity to the cyst system (lanes 13 and 14). This result shows that there is no component in the cyst 0.1 M fraction that is not in the normal trophozoite 0.1 M fraction. The amount of transcription seen with the addition of the trophozoite 0.1 M fraction to the cyst 0.3, 0.45, and 0.65 M fractions is similar to the level in the trophozoite reconstituted system (lanes 3 and 14). Thus, all cyst fractions except the 0.1 M fraction are fully active. When the other trophozoite fractions are individually substituted into the cyst system, there is only a very slight increase in transcription observed (lanes 15 to 20).

Finally, to confirm that only the 0.1 M cyst fraction was deficient in transcriptional activity and that the other cyst fractions were transcriptionally active, the individual cyst fractions were assayed for the ability to substitute for a trophozoite...
fraction in the trophozoite reconstituted system (Fig. 3, lanes 21 to 28). Each trophozoite fraction is required for transcription of the 5S RNA gene (lanes 21, 23, 25, and 27). The cyst 0.3, 0.45, and 0.65 M fractions were all able to substitute for the respective trophozoite fractions (lanes 24, 26, and 28). Only the cyst 0.1 M fraction was unable to reconstitute activity to the trophozoite system (lanes 21 and 22). From these results, it is clear that only the phosphocellulose 0.1 M KCl fraction is altered in the cyst reconstituted system. Since TFIIIA is typically found in the phosphocellulose 0.1 M fraction, it is potentially responsible for the down regulation of the 5S RNA gene during encystment.

**Active TFIIIA is not found in any of the cyst phosphocellulose fractions.** TFIIIA is found in the phosphocellulose 0.1 M fraction (flowthrough) because it is complexed with nucleic acid. Free TFIIIA elutes in the 0.65 M fraction (data not shown). Thus, the possibility remained that the cyst nuclear extract fractionated differently than the trophozoite nuclear extract and that TFIIIA was present in an active form in one of the other cyst phosphocellulose fractions. The individual cyst fractions were tested for the ability to substitute for the trophozoite 0.1 M fraction (Fig. 4). This assay showed that none of the cyst fractions were able to reconstitute activity to the trophozoite system (Fig. 4, lane 2 versus lanes 4 to 8). A slight increase in transcription is seen when the cyst 0.65 M fraction is added to the trophozoite system (lanes 3 and 7). This result suggests the presence of a small amount of active TFIIIA in this fraction (see also Fig. 9).

**5S RNA and tRNA transcription is shut off in early cyst nuclear extracts.** Since TFIIIA is a 5S RNA-specific factor, if only TFIIIA is down regulated in encysted cells, the cyst extract should still support the transcription of tRNA genes. To investigate this, the *Acanthamoeba* 5S RNA gene and a heterologous tRNA gene (*Arabidopsis thaliana* initiator tRNA\textsuperscript{Met} gene) were transcribed with either trophozoite or cyst nuclear extracts (Fig. 5). Transcription of the 5S RNA gene in the cyst nuclear extract is reduced to approximately 10% of the level in the trophozoite nuclear extract (Fig. 5, lanes 1 and 2). The tRNA gene was transcribed equally well in both the trophozoite and cyst extracts (lanes 3 and 4). The same results were obtained with the phosphocellulose-fractionated extracts (data not shown). This finding further supports the conclusion that the down regulation seen in transcription of the 5S RNA gene in *Acanthamoeba* cyst extracts is mediated by a change in the availability or activity of TFIIIA.

**The DNA binding activity of TFIIIC in cyst extracts is equivalent to that in trophozoite extracts.** An EMSA was used to evaluate TFIIIC isolated from trophozoite and encysted *Acanthamoeba* cells (Fig. 6). Various preparations of TFIIIC were assessed for the ability to bind a labeled *Arabidopsis* tRNA gene fragment and for differences in the electrophoretic mobility of the resulting DNA-protein complexes. The DNA fragment used in the TFIIIC gel shifts contained an *Arabidopsis* tRNA gene fragment from the 5’ border of the A box to the 3’ end of the gene, including the B box. The full-length *Arabidopsis* tRNA gene used to make the probe is efficiently transcribed by the *Acanthamoeba* in vitro system (Fig. 2 and 5).

Unlabeled oligonucleotides were used to compete against the labeled tRNA gene fragment for TFIIIC binding. Translations of both a B-box-containing oligonucleotide and a random control oligonucleotide were performed. The results of the assay are shown in Fig. 6. The source of TFIIIC was a phosphocellulose 0.65 M KCl fraction isolated from a trophozoite nuclear extract (lanes 1 to 5) or a cyst nuclear extract (lanes 6 to 10). The gel shifts of trophozoite and cyst TFIIIC are shown in lanes 1 and 6, respectively. For both preparations of TFIIIC, the B-box oligonucleotide could completely compete for the TFIIIC gel shift (lanes 4, 5, 9, and 10), whereas equal amounts of the random oligonucleotides did not compete for TFIIIC binding (lanes 2, 3, 7, and 8). To further demonstrate that the gel shift seen in Fig. 6 was due to TFIIIC and not some other protein present in the 0.65 M fractions, a gel shift was performed across a peak of TFIIIC activity purified on phosphocellulose with a KCl gradient. The peak of TFIIIC binding activity directly corresponded to the peak of TFIIIC transcriptional activity (data not shown). The results of these assays demonstrate a maintenance of TFIIIC DNA-binding activity during encystment. Furthermore, they do not reveal any alteration in the electrophoretic mobility of the complex attributable to phosphorylation or proteolysis, as reported during viral infection (5–7, 16, 18).

**TFIIIA DNA-binding activity is significantly reduced in the cyst 0.1 M KCl phosphocellulose fraction.** The presence of TFIIIA DNA binding activity was assayed in the trophozoite and cyst systems by using an EMSA. An end-labeled 234-bp DNA fragment containing the entire *Acanthamoeba* 5S RNA gene was used as the probe. The 0.1 M KCl phosphocellulose fractions from trophozoite and cyst extracts were assayed (Fig. 7). Strong TFIIIA DNA-binding activity is seen with the trophozoite 0.1 M KCl fraction, whereas none is seen with the corresponding cyst fraction (Fig. 7, lanes 1 and 2, respectively). TFIIIA DNA binding was effectively competed for by titration of a plasmid containing the *Acanthamoeba* 5S RNA gene (lanes 4 and 5). The vector alone (containing no insert) could not compete for TFIIIA binding (lanes 2 and 3).

**FIG. 4.** Transcriptionally active TFIIIA is not found in any of the cyst phosphocellulose fractions. The individual cyst fractions assayed in Fig. 3 were tested for the ability to substitute for the trophozoite (Troph) 0.1 M fraction in an in vitro transcription assay. The fractions (2 µl) included in each reaction mixture are indicated above each lane.

**FIG. 5.** In early cyst nuclear extracts, 5S RNA transcription is down regulated but tRNA transcription is not. The *Acanthamoeba* 5S RNA gene (lanes 1 and 2) and *Arabidopsis* tRNA\textsuperscript{Met} (lanes 3 and 4) were transcribed by both trophozoite and cyst nuclear extracts (cyst NE and Troph NE; 3 µl), as indicated.
TFIIIA fractionates in the phosphocellulose flowthrough (0.1 M KCl fraction) because of its association with nucleic acid, specifically 5S RNA. To investigate if the 5S RNA or another RNA species could be sequestering the TFIIIA so that it was unable to bind the DNA probe, RNase A treatment of the 0.1 M fractions was performed prior to EMSA analysis. RNase A treatment of the 0.1 M phosphocellulose fractions did not increase the DNA-binding activity in either the trophozoite or cyst extracts (data not shown). Therefore, it appears that TFIIIA readily exchanges between the 5S RNA and the 5S RNA gene under these assay conditions.

To further confirm that the electrophoretic mobility shift seen in Fig. 7 was due to the binding of TFIIIA, a peak of trophozoite TFIIIA activity from a DEAE-Fast Flow column was assayed (Fig. 8). TFIIIA transcriptional activity was assayed by adding the individual DEAE-Fast Flow gradient fractions to the phosphocellulose trophozoite 0.3, 0.45, and 0.65 M KCl fractions (Fig. 8A, lanes 2 to 17). Minimal transcription is seen with just the 0.3, 0.45, and 0.65 M KCl phosphocellulose fractions (lane C). Transcription is restored with the addition of the TFIIIA containing 0.1 M KCl trophozoite phosphocellulose fraction (lane 1) or the DEAE-Fast Flow gradient fractions 18 to 38 (lanes 4 to 14). An EMSA was performed across the same DEAE-Fast Flow fractions (Fig. 8B, lanes 2 to 17). The 0.1 M KCl phosphocellulose fraction was assayed as a control (lane 1). A peak of TFIIIA DNA-binding activity is seen in the DEAE-Fast Flow gradient fractions 24 to 36 (lanes 7 to 13). There is a close correlation between TFIIIA transcription activity and DNA-binding activity eluting from the DEAE-Fast Flow column (Fig. 8C). This result confirms that the DNA binding seen in the EMSA is due to TFIIIA.

TFIIIA DNA-binding activity is not present in any of the cyst phosphocellulose fractions. To ensure that TFIIIA DNA-binding activity was not present in one of the other cyst phosphocellulose fractions, an EMSA was performed across all of the trophozoite (Fig. 9, lanes 1 to 5) and cyst (lanes 6 to 10) phosphocellulose 0.1 M KCl fractions were analyzed for TFIIIA DNA-binding activity by EMSA. An end-labeled 234-bp DNA fragment containing the entire *Acanthamoeba* 5S RNA gene was used as the probe (P). The plasmid pBS+ (containing no insert) or the plasmid pBS+/5S.3, which contains the *Acanthamoeba* 5S RNA gene in pBS+, was used as the competitor at 50 ng (+) or 200 ng (+++). The probe and competitor DNAs were added simultaneously to the reaction mixtures.
alent (lanes 4 and 9). The large band seen in both the trophozoite and cyst 0.3 M KCl fractions is from nonspecific protein binding, as determined in competition assays (data not shown).

In other systems, TFIIIA is present in the 0.1 M KCl fraction from phosphocellulose because of its association with 5S RNA. This has not been shown directly for *A. castellanii* but is assumed to be true because the fractionation pattern of the nuclear extracts correlates closely with those of other systems. The small amount of TFIIIA that elutes in the 0.65 M fraction is thought to be the TFIIIA that is not associated with nucleic acid at the time of fractionation. It is interesting that the TFIIIA present in the 0.1 M KCl fraction appears to be regulated in the encysted cells. Thus, at least part of the regulatory mechanism may target the TFIIIA that is associated with the 5S RNA for degradation or inactivation through modification or by transporting out of the nucleus.

To determine if TFIIIA was being transported out of the nucleus during encystment, the cytoplasmic fraction of the trophozoite and cyst nuclear extract preparations was further purified by ammonium sulfate precipitation and phosphocellulose chromatography. An EMSA was used to assay for TFIIIA. Only a small amount of TFIIIA DNA-binding activity could be detected in the trophozoite cytoplasmic fraction, and even less was found in the cyst fraction. Since this assay may not be sensitive enough to detect low amounts of TFIIIA in dilute extracts, no definitive conclusions were drawn. Heterologous antibodies directed against *Xenopus* and yeast TFIIIA have also been tried, but they do not specifically cross-react with *Acanthamoeba* TFIIIA. Quantification of TFIIIA in the nuclear and cytoplasmic extracts will be performed when antibodies to *Acanthamoeba* TFIIIA are available.

**DISCUSSION**

We have investigated the mechanism of coordinate regulation of 5S RNA and rRNA transcription during encystment of *A. castellanii*. First, the synthesis rates of the two transcription
units were compared by pulse-labeling. The two are essentially
correlated, but 5S RNA shows a low residual transcription
rate not seen with the rRNA gene, which suggests that the two
might be regulated by different but linked mechanisms (Fig. 1).

Earlier work from this laboratory and others has shown that
rRNA transcription is regulated by direct modification of RNA
polymerase I or of a protein tightly associated with polymerase
I holoenzyme (4, 23; reviewed in reference 22). However, in
contrast to the decline in polymerase I activity, this study
revealed that the activity of RNA polymerase III in specific
transcription of 5S RNA and tRNA genes is constant (Fig. 3).
Therefore, the expression of these two RNAs is not
coordinated by modification of subunits common to the two polyn-
merases and absent from polymerase II (8, 9, 25, 26).

Analysis of the components necessary and sufficient for spe-
cific transcription of 5S RNA and tRNA genes in A. castellanii
shows that fractions with activity characteristics the same as
those found in other systems are also found in this organism.
Fractons containing factors similar to TFIIIA, TFIIIB, and
TFIIC, along with RNA polymerase III, are required for
transcription of 5S RNA, and the putative TFIIIA-containing
fraction is not needed for rRNA expression. We cannot rule
out the possibility that other components copurify with these
fractions, but at present there is no evidence for additional
required components. This is especially true for the TFIIIA
fraction, since it is not required for rRNA transcription and
thus must contain only 5S RNA gene-specific components.

We show here that Acanthamoeba 5S RNA transcription is
down regulated during encystment by alteration of the activity
of the gene-specific transcription factor TFIIIA. TFIIIA tran-
scription-stimulating and DNA-binding activities disappear in
extracts made from cells which are transcriptionally inactive. A
careful analysis showed that TFIIIA is not modified such that
it has altered fractionation characteristics but retains either of
these two activities. Therefore, it appears that either TFIIIA is
inactivated or the protein itself is not present in nuclear ex-
tracts. Attempts to analyze the cytoplasmic fraction did not
reveal TFIIIA DNA-binding activity, but we cannot be sure the
assay is sensitive enough to detect low or dilute amounts of
TFIIIA in these samples.

We are left with the problem of understanding how large
rRNA precursor and 5S RNA transcription are coordinately
regulated. One possibility, which derives from early work on

oogenesis in Xenopus laevis, is association of TFIIIA with the
product of transcription, 5S RNA (reviewed in references 31
and 32). This complex, 7S ribonucleoprotein (RNP), is trans-
ported to the cytoplasm, where an exchange of TFIIIA for a
ribosomal protein occurs, allowing TFIIIA to return to the
nucleus to participate in additional rounds of transcription.
The new 5S RNA-containing complex, 5S RNP, is then trans-
ported back to the nucleus and incorporated into the large
ribosomal subunit. The latter process requires continued
synthesis of the large rRNA precursor by RNA polymerase I.
When polymerase I is inactivated, TFIIIA is trapped in the
cytoplasm in the 7S RNP, thus coordinating rRNA and 5S
RNA transcription. Demonstration of a pool of 7S RNP in
the cytoplasm will be necessary to support this hypothesis and
will require production of homologous antibodies to Acan-
thamoeba TFIIIA.

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FIG. 9. TFIIIA DNA-binding activity is absent in the cyst phosphocellulose
fractions. An EMSA was performed across all of the trophozoite (Troph; lanes 1 to 5) and cyst (lanes 6 to 10) phosphocellulose (PC) fractions. The phospho-
cellulose fraction (2 μL) added to each reaction mixture is indicated above each lane.
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