Specific transcription of an *Acanthamoeba castellanii* 5S RNA gene in homologous nuclear extracts

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ABSTRACT

An RNA polymerase III *in vitro* transcription system has been developed from the protist *Acanthamoeba castellanii*. The system is dependent on a cloned 5S RNA gene and utilizes a nuclear extract which contains all the necessary protein components. The system is assembled from completely homologous components. Primer extension and RNA sequencing analysis confirm that the *in vitro* 5S RNA transcript is identical to the 5S RNA isolated from cells. The transcription complex forms unusually rapidly on the 5S RNA gene and is stable to challenge by excess competitor templates. Several 5' deletion mutants were constructed and indicate that the region upstream of -33 is dispensable. Deletion to +16 show the region between -33 and +16 to be required for transcription, a region outside the canonical internal control region.

INTRODUCTION

The opportunity to study molecular mechanisms of gene regulation has been facilitated by the availability of *in vitro* transcription systems. *In vitro* transcription systems for 5S RNA have been documented in a number of organisms, including *Xenopus* (1), yeast (2), *Drosophila* (3), silkworm (4), *C. elegans* (5), *Aspergillus* (6), and *Neurospora* (7). Fractionation of extracts has shown that comparable transcription factors exist in various systems, with a minimum requirement of three factors (fractions) being necessary for accurate *in vitro* transcription of 5S RNA genes (8). Work in *Xenopus* first indicated that there is a sequential assembly of transcription factors on the 5S RNA gene, which eventually leads to a stable preinitiation complex (9-11). TFIIA is the first component to bind, and associates in the absence of other factors to form a metastable binary complex covering the entire internal control region (ICR) of the 5S RNA gene (9,12-14). TFIIIC is the next factor to join the complex. Addition of TFIIIC stabilizes the TFIIA-DNA complex, forming a stable preinitiation complex (9), yet does not extend DNase I protection (15). TFIIIC does not bind specifically to the 5S RNA gene in the absence of TFIIA, but is dependent on both promoter

sequence elements, the 'A and C boxes', along with TFIIA interaction (16,17). TFIIIB is the final factor to assemble. Binding of TFIIIB to the complex is the rate limiting step in preinitiation complex formation (11). It is thought to associate with the TFIIA-TFIIIC-DNA complex via protein-protein interactions. Once the entire complex is assembled it is stable to dilution, high salt, challenge with a comparable DNA template, and able to last through multiple rounds of transcription (10). RNA polymerase III recognizes this complex, but is only loosely associated (18).

Subsequent work from other organisms has indicated additional levels of complexity. Factor C from human cell extracts has been separated into two chromatographic components, termed TFIIIC1 and TFIIIC2, both of which are required for transcription of a *Drosophila* tRNA^{Arg} gene, and the adenovirus VA1 gene (19,20). TFIIIC2 binds tightly to the B block regions of these genes and to an area surrounding the start site of the *Xenopus laevis* 5S RNA gene, apparently using different binding domains (21). Although no DNA binding activity has been shown with TFIIIC1 alone, addition of this component to the TFIIIC2-DNA complex extends protection to the A block promoter region and the transcription start site of tRNA and VA1 genes (19). From silkworm, another polymerase III factor has been identified, TFIID, which is required for transcription of class III genes (22). No single transcription factor is able to bind stably to either a tRNA or a 5S RNA gene from silkworm. Either TFIIIB or TFIIIC can bind in the presence of TFIID, which apparently had been a contaminant in earlier preparations of both TFIIIB and TFIIIC. Whether this previously unresolved factor is analogous to either of the C factors from human or whether the presence of an additional factor is required for class III genes from other organisms has yet to be determined.

To date, the most definitive study has shown that TFIIIB alone is the transcription factor, the others being assembly factors (23). In yeast, the order of assembly of the factors is the same as in *Xenopus*, but the addition of each transcription factor to the complex results in incremental extensions of DNase I protection along the 5S RNA gene (24). The upstream flanking region that is protected encompasses sequences 40-45 bp from the site of transcription initiation. The most interesting data from yeast has

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identified TFIIB as the factor which is actually involved in positioning RNA polymerase III, and which is the sole transcription initiation factor (23). TFIIA and TFIIC are involved only in assembling TFIIB at the correct position in the upstream flank. Once TFIIB is bound, TFIIA and TFIIC can be stripped off of the template with high salt or heparin. The remaining bound TFIIB can direct accurate and repeated rounds of transcription *in vitro* (23), presumably by direct contact with RNA polymerase III. This is remarkably similar to the mechanism of rRNA transcription initiation by RNA polymerase I in *Acanthamoeba* [reviewed in (25)].

Here we describe the development of a homologous 5S RNA *in vitro* transcription system from *Acanthamoeba*. Having a homologous system is important when looking at *cis* and *trans* regulatory elements because, although polymerase III systems are not as rigidly species-specific as polymerase I systems (26), variations in promoter and flanking sequences, as well as possible species-specific factors may affect the interpretation of results. The availability of an *in vitro* transcription system allows an investigation of the mechanism of coordinate regulation of 5S RNA and large precursor ribosomal RNA synthesis in *Acanthamoeba* undergoing drastic changes in cellular proliferation rate (27–29).

MATERIALS AND METHODS

Enzymes and biochemicals

Acrylamide was from Serva. Bis acrylamide, TEMED, and ammonium persulfate were from BioRad. Ultra pure urea was from Schwartz-Mann. Nucleotides were from Pharmacia P-L. α -[32 P]-UTP, γ -[32 P]-ATP, and Lightning-Plus intensifying screens were from DuPont/ New England Nuclear. Film was Fuji type RX. 5,6-[3 H]-UTP was from ICN and α -amanitin was from Boehringer Mannheim Biochemicals. Restriction endonucleases, bacterial alkaline phosphatase (BAP), T4 DNA kinase, T4 DNA ligase, RNases T1, CL3, and Phy M, and MMLV reverse transcriptase were from Bethesda Research Laboratories and were used according to manufacturers instructions. Placental ribonuclease inhibitor (RNasin) was from Promega Biotec.

Construction of 5S RNA gene templates

The clone pAc5S.1 (GenBank accession number M34003) contains an *Acanthamoeba* 5S RNA gene in a 1.2 kb *Sal* I/ *Pst* I fragment in pUC18 and had been sequenced in its entirety (30). This was subcloned to produce DNA templates containing the 5S RNA gene with varying amounts of 5' flanking sequence. pAc5S.1 was digested with *Ava* II, and a 353 bp fragment containing the 5S RNA gene with 130 base pairs of upstream sequence was filled with Klenow enzyme and cloned into the *Sma* I site of pUC18; the 5' end of the gene was oriented near the *Eco* RI site of the polycloning sequence. This clone was designated pAc5S.3. A 5S RNA 'maxigene' was constructed from pAc5S.3 by cutting with *Ava* I, producing a staggered cut with a 4 nucleotide 5' overhang, which was filled with the Klenow enzyme, and ligated with T4 DNA ligase. The resulting construct had a 4 bp insert (CCGG) near the 3' end of the coding region between base pairs 96 and 97. Subsequent digestion of the maxigene with *Eag* I within the 4 bp insert, filling with Klenow enzyme and ligating with T4 DNA ligase resulted in the construction of a maxi-maxigene which has an 8 bp insert (GGCCGGCC) between base pairs 96 and 97 of the wild type

5S RNA gene. pAc5S.3 was also digested with *Sau* 3AI and *Pvu* II to produce a 210 bp fragment which was cloned into *Bam* HI/*Sma* I cut pUC18 and designated pAc5S.3dl5'-34. This construct contains wild type sequence upstream to position -33. pAc5S.3dl5'-34 was digested with *Fsp* I (which produced a blunt-end cut at position 17 of the coding region) and *Eco* RI. This fragment was cloned into *Eco* RI/ *Sma* I cut pUC18 and resulted in a deletion to position 16 in the 5S RNA coding region, designated pAc5S.3dl5'16. The sequences of all subclones were verified by sequencing (31).

Nuclear extract preparation

This procedure is an adaptation of the method of Ohlsson and Edlund (32). Twelve liters of *Acanthamoeba* cells were grown to a density of $2-4 \times 10^6$ cells/ml (mid logarithmic phase) and harvested by centrifugation in 500 ml polycarbonate bottles at 4°C, 5,000 rpm in the Beckman JA-10 rotor for 15 minutes. The cells were washed by resuspending in 200 ml of ice cold Buffer A and centrifuged as before for 10 minutes (Buffer A: 10 mM HEPES/KOH [pH 7.9], 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF). The cell pellet was resuspended in Buffer A at two times the original packed cell volume, and the cells homogenized in a Potter-Elvehjem type homogenizer (Thomas teflon-pestle homogenizer, size C, 0.2 mm clearance) at 1700 RPM with 10 to 15 passes. When >90% of the cells were broken (as determined by phase microscopy), the cell homogenate was centrifuged at 10,000 rpm at 4°C for 10 minutes in a JA-20 rotor. The crude nuclear pellet was resuspended in 3 ml of Buffer A per 10^9 cells and transferred to Beckman Ti70.1 centrifuge tubes on ice. 4 M (NH₄)₂SO₄, pH 7.9, was added to a final concentration of 0.3 M and the nuclei lysed by gentle rocking on ice for 30 minutes. The extract was centrifuged at 36,500 rpm in the Ti70.1 rotor for 60 minutes at 2°C. The supernatants were pooled and solid ammonium sulfate added slowly (0.2 g per ml of supernatant), while gently stirring on ice. The precipitated proteins were collected by centrifugation for 15 minutes at 36,500 rpm in the Ti70.1 rotor at 2°C. The supernatant was discarded and the pellet resuspended in 1–2 ml of Buffer C (Buffer C: 50 mM HEPES/KOH [pH 7.9], 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10% glycerol). The solution was dialyzed against 2 liters of Buffer C for 6–8 hours with one change of buffer after 4 hours. The nuclear extract was then cleared by centrifugation for 1 minute at maximal speed in an Eppendorf microfuge at 4°C. The extracts were divided into aliquots, and stored in liquid nitrogen until further use. The protein concentration was usually 15–20 mg/ml with approximately 8 units/ml of RNA polymerase III (33). The remaining RNA polymerase in the extract was polymerase II: there was about 60% polymerase II and 40% polymerase III in these extracts. No RNA polymerase I was detected in six different nuclear extracts.

In vitro transcription assay

The standard assay (25 μ l final volume) contained the following: 50 mM HEPES/KOH (pH 7.9), 100 mM KCl, 7.5 mM MgCl₂, 2% glycerol (from the nuclear extract), 1 mM dithiothreitol, 600 μ M each of ATP, CTP, and GTP, 50 μ M UTP, 5 μ Ci of α -[32 P]-UTP (3000 Ci/mmol), 100 ng of supercoiled plasmid DNA and 5 μ l of nuclear extract. The reactions were incubated at 25°C for 30 minutes and terminated by the addition of 25 μ l stop buffer [0.4% SDS, 100 mM sodium acetate (pH 5.2), 1 mg/ml *E. coli* tRNA] and 50 μ l of TEN buffer (50 mM Tris-

HCl [pH 7.9], 1 mM EDTA, 100 mM NaCl). The reactions were phenol: chloroform: isoamyl alcohol (100:100:1) extracted once, chloroform: isoamyl alcohol (100:1) extracted once, and the aqueous fraction transferred to a new tube and ethanol precipitated for 30 minutes at -70°C . The reactions were then centrifuged for 10 minutes at maximal speed in an Eppendorf centrifuge, the supernatant discarded, and the pellets dried briefly under vacuum. The pellets were resuspended in $5\ \mu\text{l}$ of 95% deionized formamide, 10 mM EDTA, 0.1% Bromophenol Blue (BPB), 0.1% Xylene Cyanol FF (XC), and analyzed on an 8% (8%T, 5%C) polyacrylamide gel containing 8 M urea. Quantitation of RNA synthesis was performed by Cerenkov counting of the excised, radioactive 5S RNA band.

RNA polymerase preparation

RNA polymerase III used to supplement some reactions (eg. Fig. 1) was purified by our previously published procedure (34) except omitting the final glycerol gradient step.

Primer extension/RNA sequencing

RNA was resuspended in $10\ \mu\text{l}$ of Annealing Buffer (250 mM KCl, 10 mM Tris-HCl [pH 8.3]). One μl (100 pg) of 5'-[^{32}P]-labelled primer was added, mixed, and incubated at 80°C for 3 min. Annealing was performed by incubating the reaction at 45°C for 120 min. For each RNA sample, a $10\ \mu\text{l}$ primer extension reaction was set up as follows: $1\ \mu\text{l}$ $10\times\text{RT}$ ($10\times\text{RT}$: 340 mM Tris-HCl [pH 8.3], 60 mM MgCl_2 , 50 mM DTT), $2\ \mu\text{l}$ annealed RNA + primer, $2\ \mu\text{l}$ $5\times\text{dNTP}$ ($5\times\text{dNTP}$: 1.0 mM each dATP, dGTP, dTTP, dCTP), $0.5\ \mu\text{l}$ Actinomycin D (1 mg/ml), $3.5\ \mu\text{l}$ H_2O , and $1\ \mu\text{l}$ MMLV Reverse Transcriptase (BRL; 200 units/ μl). Each reaction was incubated at 37°C for 30 minutes and the reaction stopped with $5\ \mu\text{l}$ loading buffer (95% formamide, 10 mM EDTA, 0.1% each XC and BPB). Lanes of an 8% sequencing gel were loaded with 3–4 μl from each reaction.

RESULTS

Specific transcription of the 5S RNA gene utilized a nuclear extract prepared by a modification of the method of Ohlsson and Edlund (32). We have attempted to make nuclei which were 'cleaner' than the crude preparation used here, by several techniques, but none of these extracts were active. Further, the characteristics of the homogenizer used to break the cells seems to be critical to the preparation of active extracts; homogenization with a dispaix-type machine was completely ineffective. We rule out the presence of inhibitors in the cleaner nuclei because the inactive extracts are not inhibitory when mixed with the active extracts (data not shown).

Transcription is dependent upon RNA polymerase III

The α -Amanitin sensitivity of transcription indicates that the system is dependent on RNA polymerase III. As shown in figure 1, low ($2\ \mu\text{g}/\text{ml}$) concentrations of α -amanitin had no effect, but high ($600\ \mu\text{g}/\text{ml}$) concentrations completely inhibited transcription of 5S RNA. α -Amanitin is a specific inhibitor of RNA polymerases, with different concentrations of the toxin inhibiting a particular class of enzyme (35). In *Acanthamoeba*, as in vertebrates, RNA polymerase I is insensitive to the toxin, RNA polymerase II is inhibited by $2\ \mu\text{g}/\text{ml}$, and RNA polymerase III is inhibited by $600\ \mu\text{g}/\text{ml}$ (33).

The *in vitro* system initiates faithfully

To determine whether the 5S RNA produced *in vitro* initiated correctly, primer extension reactions were performed on RNA made *in vitro* or isolated from cells and compared. RNA either generated *in vitro* using as template the 5S RNA wild type gene (Figure 2, lane 8), the maxigene (Figure 2, lane 9) or the maxi-maxigene (Figure 2, lane 10), or *in vivo* RNA from whole cells (Figure 2, lane 1) or from the nuclear extract (Figure 2, lanes 2 and 7) was isolated and purified as described in Materials and Methods. A 5S RNA-specific 15 nucleotide long primer which complements positions 97 to 111 of the wild type 5S RNA was labelled on its 5' end by phosphorylation with ^{32}P , annealed to the isolated RNAs and extended with reverse transcriptase. Using the same, but unlabelled (*i.e.* with a 5'-OH group) primer, a sequence ladder from the wild type 5S RNA gene was created and electrophoresed next to the primer extension products. In these assays, electrophoretic mobilities of the sequencing ladder products are slower, by 1/2 nucleotide, than the primer extension products because of the 5'-phosphorylation difference in the primers used. This difference demonstrates that the method is precise to within 1/2 nucleotide. Both the isolated *in vivo* 5S RNA (Figure 2, lane 1), the 5S RNA endogenous to the nuclear extract (Figure 2, lanes 2 & 7) and the *in vitro* transcript from the wild type template (Figure 2, lane 8, note the increased intensity due to endogenous plus *in vitro* products) produce primer extension products of identical lengths, which correspond to the 5' end of 5S RNA, based upon its primary sequence (36,37). The maxigene and maxi-maxigene transcripts were found to contain the same 5' end as the wild type transcript. The full length primer extension product for the maxigene is 4 nt longer (Figure 2, lane 9, marked with an asterisk; note also the extension product for the endogenous RNA) since the primer anneals just downstream of the insert, and is 8 nt longer for the maxi-maxigene product (Figure 2, lane 10, marked with an asterisk). Faithful initiation was also verified by confirming identical 5' sequences of the endogenous and maxigene products by including dideoxynucleotides in the primer extension reactions (data not shown), and by comparison of the partial RNase digestion patterns of isolated *in vivo* and *in vitro* RNAs. Partial digestion of 5'-[^{32}P]-end labelled *in vivo* and *in vitro* RNAs with RNases T1, PhyM, and CL3 yielded identical patterns (data not shown).

Figure 1. *In vitro* transcription assays were performed as described in Materials and Methods with varying α -amanitin levels. Lane 1 has no α -amanitin, lane 2 has low α -amanitin ($2\ \mu\text{g}/\text{ml}$), lane 3 has high α -amanitin ($600\ \mu\text{g}/\text{ml}$), lane 4 is the same as lane 1 with the addition of 60 milliunits RNA polymerase III.

Figure 2. Primer extension analysis of *in vivo* and *in vitro* 5S RNAs. 5S RNA was isolated from log phase *Acanthamoeba* cells (*in vivo*) or from the standard transcription assay using wild type DNA (wt), the maxigene (maxi) or maxi-maxigene (maxi-maxi) as template. The RNAs produced in these *in vitro* reactions, as well as the endogenous 5S RNA present in the nuclear extract (endo) were phenol extracted and ethanol precipitated and subjected to primer extension analysis as described in METHODS. Sequence ladders (G, A, T, C, and C) were produced from the wild type gene using the same primer, but unphosphorylated. The arrowhead indicates the first coding nucleotide, C, lined up with the sequences of the DNA and 5S RNA, which starts with a G ([1031,2156]). The asterisks next to the primer extension product bands in the maxi and maxi-maxi lanes indicate the products which are 4, and 8 nucleotides longer, respectively, than the wild type (wt). Note that the primer extension products electrophorese 1/2 nucleotide faster than the sequencing ladder because the latter primer is unphosphorylated.

Optimum conditions

The conditions for 5S RNA transcription were adjusted for maximal transcript production. Optimal KCl concentration is 75 mM (Figure 3A). The system functions efficiently from 75 to 125 mM, and 100 mM is used routinely. Magnesium ion concentration was also varied; activity was approximately equal from 5 to 10 mM (Figure 3B). 7.5 mM is used in the standard assay. When 5S RNA template (pAc5S.1) is used alone in the assay, the DNA concentration was found to be optimal from 4 $\mu\text{g/ml}$ to 24 $\mu\text{g/ml}$ (100–600 ng of template in a 25 μl reaction). This corresponds to a molar concentration of 1.55–9.3

Figure 3. Determination of optimum transcription conditions. **A.** Determination of optimum KCl concentration. The standard transcription assay was performed with varying KCl concentrations as shown. **B.** Determination of optimum MgCl_2 concentration. The standard transcription assay was performed with varying MgCl_2 concentration as shown. **C.** The effect of varying concentrations of DNA template were examined. The supercoiled plasmid template pAc5S.1, a pUC18 derivative with one copy of the *Acanthamoeba* 5S RNA gene, was varied as shown, in the absence of other added DNA. **D.** The effect of bulk DNA concentration. The template concentration (pAc5S.1) was varied as shown either in the absence of additional DNA (filled circles) or in the presence of pUC18 (inverted triangles) to make up a constant total DNA concentration of 8 $\mu\text{g/ml}$.

nM for the pAc5S.1 template (Figure 3C; Note that the x-axis is a logarithmic scale). In crude extracts, nonspecific DNA-binding proteins inhibit transcription, and part of the role of the added DNA appears to be to complex these nonspecific proteins (38). This role can be carried out by nontemplate DNA. Figure 3D shows a titration of template DNA (pAc5S.1) in the presence of sufficient pUC18 to maintain the total DNA concentration at 8 $\mu\text{g/ml}$. Optimum transcription is reached by 3–4 $\mu\text{g/ml}$, but template activity is higher at lower template concentrations than when no bulk DNA is added. The effect is not striking, however, except at very low template concentrations, suggesting that there are not significant amounts of inhibitors in these extracts [cf. data in (38)]. Small differences in these template optima have been observed between different nuclear extracts.

Most 5S RNA *in vitro* systems, for example *Drosophila* (3), require a long lag time for assembly of the factors before transcription ensues (approximately 45 minutes). A time course study for the *Acanthamoeba* system was performed (Figure 4). The 5S RNA product begins to appear between 2½ and 5 minutes, indicating that a short lag time is necessary for assembly of the transcription complex. Preincubation of the extract with the DNA template reduces the lag time to less than one minute (data not shown). 5S RNA transcription is approximately linear up to 60 minutes and continues to increase until 90 minutes, whereafter a decrease in product is seen. This experiment was repeated with another DNA template (pAc5S.3; 130 bp of upstream sequence), and the same result was obtained (data not

Figure 4. Time course of transcription. The standard transcription assay was performed for the various times indicated.

Figure 5. Comparison of template supercoiling on transcriptional efficiency. Linear and supercoiled templates were compared in the standard assay. *Lane 1:* 4 μ g/ml covalently closed, supercoiled pAc5S.1. *Lane 2:* 4 μ g/ml linearized pAc5S.1. The template was linearized with *Sall*, which contains a single site approximately 600 bp from the 5S gene.

shown). The standard assay, as described previously using pAc5S.1 as template, is routinely incubated for 30 minutes.

The effect of linear vs. supercoiled template was also examined (Figure 5). A linearized DNA template has 20% the activity of a circular supercoiled template, similar to the results of Brown and Gurdon (1978).

Transcription requires sequence 5' of the canonical internal control region (ICR)

The first analysis of a 5S RNA gene promoter from a eukaryote, *Xenopus laevis*, reported that the entire promoter was internal to the transcription unit, extending no further upstream than about base pair 45 (12,13). Subsequently, a number of groups have reported that 5' flanking regions affect transcription of 5S RNA genes (4,39,40). Therefore, several different clones of the *Acanthamoeba* 5S RNA gene having 5' flanking sequence varying from 4 kb to 33 base pairs, were compared for transcriptional efficiency. None of the clones, which were used in the transcription assay at equimolar concentrations, showed significant differences in transcriptional efficiency (Figure 6A). Often, flanking sequence requirements are not detectable until the templates are assayed under non-optimal conditions (4). Whether the *Acanthamoeba* deletions were assayed under optimal conditions (100 mM KCl) or non-optimal conditions (KCl concentration of 150 mM, where transcription is reduced to approximately 50% from optimal, data not shown), no differences in transcription were seen. Therefore, if there is any upstream

Figure 6. Transcription with templates containing various 5' sequence deletions. **A.** Deletions in the 5' flanking sequence. The amount of remaining upstream sequence is indicated above each lane. **B.** Deletion of all 5' sequence plus the first 15 coding base pairs. *lane 1.* The 5' deletion extends to -130. *lane 2.* The 5' deletion extends into the transcription unit to position 16.

sequence requirement, it must be found within the 33 base pairs upstream of the start site. A TATA box and a pentamer motif (30) are both found in the 33 base pairs immediately preceding the gene. It is not yet known what function, if any, these sequences have in the *Acanthamoeba* system. However, a template deleted to position 16 within the transcription unit (pAc5S.3d15'+16) was not transcriptionally active (Figure 6B). This deletion has an endpoint considerably upstream of the canonical ICR, which extends from position 48 to 100 in *Acanthamoeba*, based upon the consensus compiled by Pieler and coworkers (16). This suggests significant differences from the *Xenopus* 5S RNA promoter.

DISCUSSION

A 5S RNA *in vitro* transcription system has been developed from *Acanthamoeba* and the various parameters affecting transcription have been examined. α -Amanitin sensitivity studies and 5' end analysis by primer extension coupled with product size and sequence determination show that both correct initiation and termination by RNA polymerase III are occurring in the system. Conditions for optimal transcription do not differ significantly from systems developed previously from other organisms. One

possible exception is the low presence of DNA-binding inhibitors in the *Acanthamoeba* nuclear extract (Figure 3C and D).

Kassavetis *et al.* (23) have identified in yeast what appears to be the fundamental mechanism of initiation for 5S RNA and tRNA genes. Based upon this study, the promoter appears to consist of four protein binding domains: a downstream site, within the coding region, for TFIIB, a site immediately upstream of the first for TFIIC, a site upstream of the coding region for TFIIB and a domain between the TFIIB and TFIIC sites which interacts with polymerase III (see Introduction). The limited deletion analysis carried out with the *Acanthamoeba* 5S RNA gene is consistent with, but does not prove, a similar promoter arrangement. In yeast (23), subsequent to assembly, TFIIB and TFIIC can be removed from the template by high salt treatment and the remaining TFIIB can catalyze multiple rounds of initiation. This mechanism is fundamentally identical to that identified for initiation of large precursor ribosomal RNA in *Acanthamoeba* (25). These similarities will facilitate a detailed analysis of the regulation mechanism of 5S RNA transcription and a comparison with the mechanism of coordinate regulation of larger rRNA precursor synthesis by RNA polymerase I (27–29).

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