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Ribosomal RNA transcription: Proteins and DNA sequences involved in preinitiation complex formation

(RNA polymerase I/promoter/BAL-31 deletion mutants/*in vitro* transcription/*Acanthamoeba castellanii*)

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ABSTRACT **An *in vitro* transcription system consisting of partially purified transcription initiation factor(s) and purified RNA polymerase I from *Acanthamoeba castellanii* was used to study the mechanism of faithful initiation of ribosomal RNA transcription. Formation of a preinitiation complex between one or several auxiliary transcription proteins and the DNA template in the absence of RNA polymerase I was demonstrated. A series of 3′- and 5′-deletion mutants of the template was used in prebinding competition experiments and provided evidence for three distinct functional regions of the promoter: core motif A interacts with the transcription initiation factor(s) and is required for faithful transcription; the start motif is required for transcription, but it can be deleted without affecting the binding of transcription initiation factor(s); and motif B stabilizes preinitiation complex formation (in addition to core motif A), but it is dispensable for faithful initiation of transcription.**

In prokaryotic systems, transcription involves interaction between a single RNA polymerase and the DNA template. The polymerase holoenzyme alone is capable of recognizing the promoter for each transcribed gene. In contrast, three different RNA polymerases are needed to transcribe the full complement of genes in eukaryotes. Each polymerase is involved in transcribing a distinct set of genes. Despite the complex subunit architecture of the eukaryotic polymerases (1), auxiliary proteins (transcription initiation factors; TIFs) unique for each polymerase are required in addition to polymerase for promoter recognition. In polymerase III (2–4) and II (5) systems, the TIFs bind to the promoter DNA, forming a stable preinitiation complex, which is subsequently recognized by the polymerase. Using crude cell-free extracts containing the TIFs and RNA polymerase I (RNAP-I), Wandelt and Grummt (6) and Cizewski and Sollner-Webb (7) demonstrated the formation of stable complexes with ribosomal DNA templates in a mouse system, and recently Miesfeld and Arnheim (8) have presented similar data for human extracts. We have shown previously (9) that ribosomal RNA transcription *in vitro* is species specific, and Mishima *et al.* (10) presented evidence that this specificity resides in one of the TIFs. Miesfeld and Arnheim (8) demonstrated that it is the species-specific TIF that is involved in preinitiation complex formation. Evidence was also reported supporting the notion that RNAP-I was not needed for preinitiation complex formation (8). However, since the polymerase preparation used in their study also formed a stable complex with the DNA template, the role of RNAP-I in complex formation was unclear. In contrast, we have used a partially purified TIF preparation and highly purified RNAP-I from the protozoan *Acanthamoeba castellanii*, incapable of specific initiation *in vitro* (11). Using these preparations, we demonstrate that, in analogy to polymerase II and III systems, the TIFs first bind to the promoter in the absence of RNAP-I to form a stable preinitiation complex. RNAP-I then binds to this complex to form an initiation complex capable of *de novo* synthesis of a faithful RNA transcript. In addition, we have used a series of deletion mutants to identify the template sequences involved in complex formation.

The core promoter (which we define as the minimal DNA sequence required for faithful *in vitro* transcription) was shown to consist of two sequence motifs. One motif is proximal to the start site and is required for transcription but not for TIF binding. Two upstream sequences are involved in TIF binding. Only one is required for transcription and transiently interacts with TIF; the second is necessary for stable preinitiation complex formation.

MATERIALS AND METHODS

DNA Templates. A 74-base-pair (bp) *Xma* III-generated fragment containing the initiation region for the ribosomal RNA gene of *Acanthamoeba* was cloned into the *Xma* III site of pBR322. This ribosomal DNA fragment, extending from −55 to +19, was inserted in both orientations to produce the clones pSBX60 and pSBX60i. In the following experiments, the plasmids were linearized with different restriction enzymes (Bethesda Research Laboratories) to produce RNA runoffs of diverse size in the cell-free transcription system (Fig. 1).

pSBX60 (3′ deletions) or pSBX60i (5′ deletions) were cut with *Nru* I (Bethesda Research Laboratories), and the linearized templates were digested with "slow" BAL-31 (International Biotechnologies, New Haven, CT) for 20–25 min. The resulting mixture of deleted DNAs were treated with T4 DNA ligase and used to transform *Escherichia coli* strain SK1592. The extent of deletion in the resulting mutants was determined by DNA sequence analysis.

***In Vitro* Transcription.** S100 extracts were prepared according to Weil *et al.* (12) from logarithmic phase *A. castellanii* cells. The extracts were partially purified by chromatography on phosphocellulose (P11, Whatman). S100 (15 ml) dialyzed against buffer A (20 mM Tris·Cl, pH 7.9/0.2 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol) containing 0.1 M KCl was applied to a phosphocellulose column (2.5 × 5.0 cm) equilibrated with 0.1 M KCl in buffer A. The column was washed with 5 vol of buffer A containing 0.1 M KCl, then fractionated with 3 vol of buffer A containing 0.4 M KCl, 3 vol of buffer A containing 0.6 M KCl, and 3 vol of buffer A containing 1.0 M KCl. Fractions were pooled according to absorbance at 280 nm, dialyzed at 4°C for 4 hr against buffer A containing 0.1 M KCl, and assayed for TIF and RNAP-I activity. The 0.6 M KCl fraction contained the TIF(s) needed for faithful initiation in the *Acanthamoeba*

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Abbreviations: TIF, transcription initiation factor; RNAP-I, DNA-dependent RNA polymerase I; bp, base pair(s).

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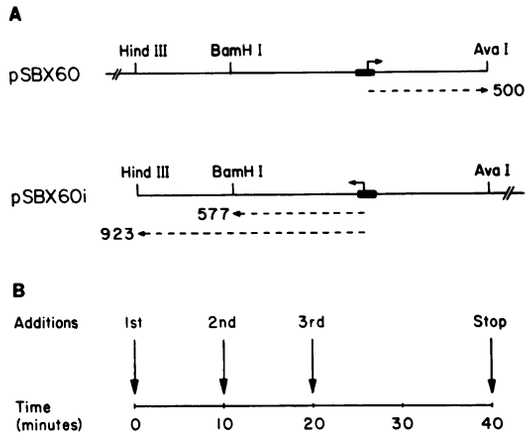

FIG. 1. DNA templates and outline of experimental protocol used in prebinding competition assays. (*A*) Diagram of pSBX60i and pSBX60 and the runoff RNAs produced. (*B*) Diagram of the sequence of additions used in the prebinding competition assays shown in Figs. 2*C*, 3, 4, and 5.

cell-free system. This TIF extract was quick-frozen and stored in liquid nitrogen.

Transcription activity was estimated under the optimum conditions determined in this laboratory for RNA runoff transcription (11). The standard transcription reaction was in a 25-μl final volume containing 10 mM Tris·HCl, pH 7.9/0.1 mM EDTA/0.5 mM dithiothreitol/10% (vol/vol) glycerol/7.5 mM $MgCl_2$/150 mM KCl/0.6 mM each ATP, UTP, GTP/2.5 μM CTP/2.5 μCi of [α-^{32}P]CTP (specific activity, 2500 Ci/mmol; 1 Ci = 37 GBq)/0.1–0.5 μg of linearized plasmid DNA/6 μl of TIF extract/20 milliunits of heparin-Sepharose purified RNAP-I (13). Incubation was at 25°C for 20 min and RNA was extracted and analyzed as described (11).

Competition Prebinding Assay. The first DNA template (0.15 μg) was preincubated for 10 min at 25°C in an 18-μl reaction volume containing 13.9 mM Tris·HCl, pH 7.9/0.14 mM EDTA/0.69 mM dithiothreitol/13.9% (vol/vol) glycerol/10.4 mM $MgCl_2$/150 mM KCl/6 μl of TIF extract/20 milliunits of RNAP-I. The second template (0.15 μg in 1 μl) was then added and the mixture was allowed to further incubate at 25°C for another 10 min. Synthesis of RNA was initiated by the addition of the nucleoside triphosphates and KCl to maintain the KCl concentration at 150 mM. Final reaction volume was 25 μl at the conditions of the standard transcription reaction. The first DNA template was allowed to preincubate with the TIF extract and exogenous RNAP-I in all cases except Fig. 2*C*, where preincubation was carried out in the presence of either TIF extract or RNAP-I alone. This was then followed by the addition of the second DNA template and initiation with nucleoside triphosphates and either RNAP-I or TIF extract.

RESULTS

Transcription of Deleted DNA Templates. A 74-bp *Xma* III fragment extending from −55 to +19 was excised from a clone of the ribosomal DNA from *A. castellanii* (11, 14) and subcloned into the *Xma* III site of pBR322. This clone was shown to support faithful *in vitro* transcription in a homologous RNAP-I-dependent system (11). To further delineate the core promoter sequence, a series of 5′ and 3′ deletions extending into the *Xma* III region were produced by BAL-31 digestion. These were tested for their ability to support correct initiation in the *in vitro* system. Deletions from the 5′ end extending to −32 were fully active (see Fig. 4, lanes 2–8, transcripts labeled A). Deletions extending from the 5′ end to −26 or further downstream are inactive (see Fig. 4, lanes 9–12, transcripts labeled A). Therefore, the 5′ border of the core promoter is between −31 and −26. To obviate the possibility of excluding a required nucleotide pair, we use the convention of including all of the possible required sequence and, thus, define the upstream border of the promoter as −31. Deletions from the 3′ end extending to +9 were fully active, while a deletion to −13 was transcriptionally inactive (see Fig. 3, transcripts labeled A). Thus, by using the convention described above, the upstream and downstream borders of the core promoter are −31 and +8, respectively. However, these results do not distinguish one large functional region from several functionally distinct sequence motifs. Therefore, a competition prebinding assay was used to identify those regions of the core promoter involved in binding of protein components of the transcriptional apparatus.

Preincubation of the DNA Template with Transcription Initiation Factor in the Absence of RNAP-I. Faithful transcription is assayed *in vitro* by a runoff assay (11). TIF, RNAP-I, and a DNA template that has been truncated at a known distance from the start site are incubated in the presence of ^{32}P-labeled nucleoside triphosphates. The RNA products are isolated, separated by electrophoresis, and visualized by autoradiography. Transcriptional activity of templates truncated by different restriction enzymes can be distinguished by the size of the runoff RNAs they produce, even when both DNAs are in the same reaction mixture. For example, in Fig. 2*C*, lane 1 contains only DNA A, lane 2 contains only DNA B, and lane 3 contains both templates. The high molecular weight smear observed above the runoff RNAs of interest (e.g., Fig. 2*C*, lanes 4 and 7; more prominent in Fig. 3) results from nonspecific transcription. Its amount is variable and depends on the template used and the preincubation components (crude TIF preparations increase it), but in the presence of excess RNAP-I, it appears to have no influence on the efficiency of faithful initiation.

A variation of the runoff assay is the competition prebinding assay in which one DNA is preincubated with transcriptional components prior to the addition of a second DNA. These assays have been interpreted by us and others (2, 4–8) as measuring the ability of a DNA template to form a stable complex with transcription factor(s). According to this interpretation, when an excess of a single DNA template is preincubated with TIF and RNAP-I in the absence of nucleoside triphosphates, the template sequesters all of the available TIF into an initiation complex. If a second DNA template is subsequently added, it is unable to form a transcriptionally active complex, and addition of nucleoside triphosphates results in transcription of only the first DNA (Fig. 2*C*, lanes 6 and 9). We found that 0.15 μg of DNA was just sufficient to sequester all of the TIF in our standard assay by adding increasing amounts of the first DNA until an equal amount of the second DNA was no longer transcribed (data not shown). We also found that a stable TIF–DNA complex formed within 5–10 min by estimating the preincubation time required for the first template to prevent transcription of the second (see Fig. 2*A*). This complex was stable for >1 hr, even in the presence of a competing template (Fig. 2*B*). For genes transcribed by polymerase II and 5S RNA genes (transcribed by polymerase III), it has been demonstrated that preinitiation complex formation between one or several TIFs and the DNA template occurs in the absence of polymerase. Therefore, we tested the ability of the partially purified TIF-I preparation alone and the RNAP-I alone, to support preinitiation complex formation. The TIF preparation used in these experiments was incapable of producing measurable runoff products in the standard assay and was thus free of RNAP-I activity. Fig. 2 shows that when the DNA template was preincubated with TIF, challenged with a second DNA, followed by addition of purified RNAP-I, only the first DNA was transcriptionally active (Fig. 2*C*, lanes 4 and 7). Howev-

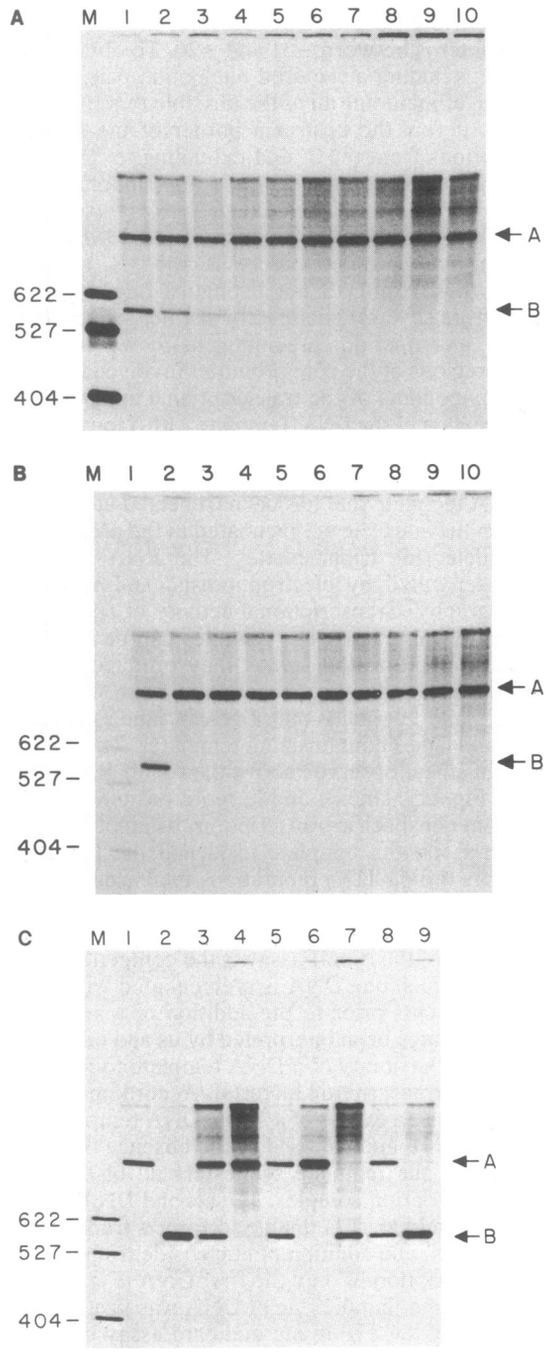

FIG. 2. Formation and stability of preinitiation complexes in the absence or presence of RNAP-I. The autoradiograms show RNA runoff products transcribed from pSBX60i/*Hin*dIII (labeled A; 923 nucleotides) or from pSBX60i/*Bam*HI (labeled B; 577 nucleotides). (*A*) Formation: time between addition of first DNA template (labeled A) and second template (labeled B) to a reaction mixture containing the TIF extract and RNAP-I was varied. Nucleoside triphosphates were added 10 min after the addition of the second template to initiate transcription. Lanes: 1, both templates added simultaneously; 2, 30 sec; 3, 1 min; 4, 2 min; 5, 3 min; 6, 5 min; 7, 10 min; 8, 15 min; 9, 30 min; 10, 60 min. (*B*) Stability. After preincubation of the first template with the TIF extract and RNAP-I for 10 min, the time was varied between the addition of the second template and the nucleoside triphosphates. Lanes: 1, control, DNA templates added simultaneously to TIF extract and RNAP-I, and transcription initiated after a 10-min preincubation by addition of nucleoside triphosphates; 2, nucleoside triphosphates added simultaneously with the second template. In lanes 3–10, time between addition of the second DNA and nucleoside triphosphates was as follows: 3, 1 min; 4, 3 min; 5, 5 min; 6, 10 min; 7, 15 min; 8, 30 min; 9, 45 min; 10, 60 min. (*C*) Order of protein addition. Preinitiation complex formation was assayed. Preinitiation was carried out in the presence of different transcriptional proteins to determine those components necessary for stable preinitiation complex formation. Lane 1, (1st) DNA A/TIF extract/RNAP-I; (2nd) no DNA; (3rd) NTPs. Lane 2, (1st) DNA B/TIF extract/RNAP-I; (2nd) no DNA; (3rd) NTPs. Lane 3, (1st) DNA A/DNA B/TIF extract/RNAP-I; (2nd) no DNA; (3rd) NTPs. Lane 4, (1st) DNA A/TIF extract; (2nd) DNA B; (3rd) NTPs/RNAP-I. Lane 5, (1st) DNA A/RNAP-I; (2nd) DNA B; (3rd) NTPs/TIF extract. Lane 6, (1st) DNA A/TIF extract/RNAP-I; (2nd) DNA B; (3rd) NTPs. Lanes 7, 8, and 9, same as lanes 4–6 except the order of addition of DNAs A and B is reversed. Lanes M, molecular weight markers in nucleotides.

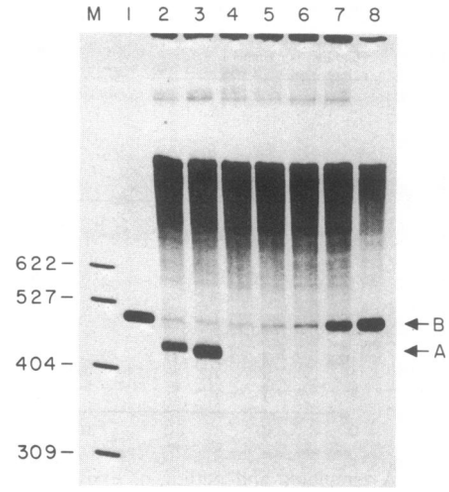

FIG. 3. Prebinding competition between 3′-deletion mutants and wild-type DNA templates. Preinitiation complex formation was assayed. Autoradiogram shows RNA runoff products transcribed from 3′-deletion mutants/*Ava* I (labeled A; variable lengths) and from pSBX60/*Ava* I (labeled B; 500 nucleotides). Deletions upstream of +9 do not yield transcripts. Lane 1, DNA B transcribed in the absence of competitor. In all remaining lanes, the 3′-deletion mutant terminating at the position indicated below was preincubated with TIF extract/RNAP-I. The second addition was DNA B and the reaction was initiated by addition of NTPs. Lanes: 2, 3′dl +14; 3, 3′dl +9; 4, 3′dl −13; 5, 3′dl −16; 6, 3′dl −19; 7, 3′dl −27; 8, 3′dl −29. Lane M, molecular weight markers in nucleotides.

er, preincubation of the first DNA with RNAP-I, followed by the addition of a second template and TIF, resulted in equal transcription of both templates (Fig. 2*C*, lanes 5 and 8). Thus, since the TIF sequestered the first DNA in the absence of RNAP-I, we concluded that the TIF formed a preinitiation complex with the template, which was later joined by the polymerase.

Competition Between Deleted Templates and Wild-Type DNA. The competition prebinding assay allows evaluation of template regions that are involved in TIF binding. If 5′- and 3′-deletion mutants are used as the first DNA, only those capable of stably binding the TIF will prevent transcription of a second wild-type DNA. Fig. 3 shows the results of an experiment in which various 3′ deletion mutants (whose runoff product is labeled A in the figure) were preincubated with TIF and RNAP-I and then challenged with a second DNA (labeled B). Deletions from the 3′ end extending to −19 (lanes 2–6) all sequester the TIF and prevent transcription from the second DNA. Deletions extending to −27 or beyond (lanes 7 and 8) do not form complexes capable of preventing transcription of the second DNA. Fig. 4 shows that 5′ deletions (labeled A) extending to −48 effectively bind the TIFs into a preinitiation complex (lanes 2–4), whereas deletions to −40, −37, −35, −32 (lanes 5, 6, 7, and 8, respectively), and beyond are permissive for transcription of the second template (labeled B). Thus, these experiments show that the region involved in preinitiation complex formation is

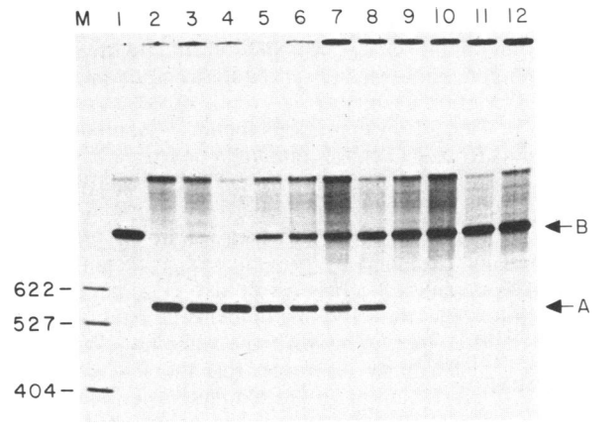

FIG. 4. Prebinding competition between 5′-deletion mutants and wild-type DNA templates. Preinitiation complex formation was assayed. Autoradiogram shows RNA runoff products transcribed from 5′-deletion mutants/*Bam*HI (labeled A; 577 nucleotides) and from pSBX60i/*Hin*dIII (labeled B; 923 nucleotides). Lane 1, DNA B transcribed in the absence of competitor. In all remaining lanes, the 5′-deletion mutant terminating at the position indicated below was preincubated with TIF extract/RNAP-I. The second addition was DNA B and the reaction was initiated by addition of NTPs. Lanes: 2, 5′dl −55; 3, 5′dl −53; 4, 5′dl −48; 5, 5′dl −40; 6, 5′dl −37; 7, 5′dl −35; 8, 5′dl −32; 9, 5′dl −26; 10, 5′dl −22; 11, 5′dl −20; 12, 5′dl −15.

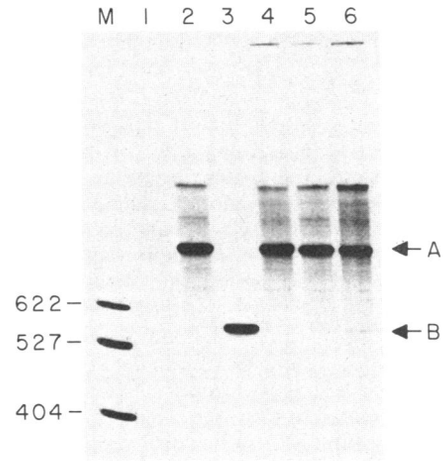

FIG. 5. Prebinding competition between three distinct DNA templates. Preinitiation complex formation was assayed. DNA A was pSBX60i/*Hin*dIII (labeled A; 923 nucleotides). DNA B was pSBX60i/*Bam*HI (labeled B; 577 nucleotides). Lanes: 1, 3′dl −29 transcribed alone (transcriptionally inactive); 2, DNA A transcribed alone; 3, DNA B transcribed alone; 4, (1st) 3′dl −29/TIF extract/RNAP-I, (2nd) DNA A, (3rd) no DNA, (4th) NTPs; 5, (1st) DNA A/TIF extract/RNAP-I, (2nd) DNA B, (3rd) NTPs; 6, (1st) 3′dl −29/TIF extract/RNAP-I, (2nd) DNA A, (3rd) DNA B, (4th) NTPs. Lane M, molecular weight markers in nucleotides.

somewhere between −47 and −20. It is noteworthy that these upstream and downstream borders determined by the competition prebinding assay are significantly different from the borders determined by transcriptional activity (−31 to +8).

A comparison of the transcription and the binding/competition studies of the 5′ deletions suggests that there are two sequence domains involved in TIF interaction: One (core motif A, somewhere between −20 and −31) is needed for faithful initiation of transcription and, therefore, must interact at least transiently with a TIF. The second, more upstream motif (motif B, between −32 and −47), is necessary in addition to core motif A to form stable preinitiation complexes. We tested whether motif B alone could form a stable complex with a TIF, thus sequestering the component needed to stabilize the complete preinitiation complex. A 3′ deletion (3′dl −29) containing motif B, but not core motif A, was preincubated with TIF and RNAP-I; then a second wild-type DNA was added and allowed to bind TIF(s). If the stabilizing component had been stably bound by the 3′dl −29, this second DNA (labeled A in Fig. 5) should not be able to form a stable preinitiation complex. Thus, when a third wild-type template (labeled B) is added, both should be equally transcribed. However, template A was able to form a stable preinitiation complex and prevented transcription of template B (Fig. 5, lane 6) indicating that 3′dl −29 containing only motif B is incapable of tightly binding a component needed to stabilize the preinitiation complex.

DISCUSSION

Using a series of 5′- and 3′-deletion mutants of the core promoter for ribosomal RNA and two different assay techniques, evidence for several sequence regions with distinct functions has been obtained. One of these regions is absolutely required for transcription (the start motif, Fig. 6) but functions in some step other than TIF binding. This region is defined by two sets of functional tests: transcription assays show that 3′ deletions to +9 are active, but a deletion to −13 is not. Thus, the sequence required for transcription extends downstream to at least −13, and perhaps as far as the first 8 nucleotide pairs of the external transcribed spacer. However, competition prebinding assays show that 3′ deletions to −19 are fully capable of TIF binding. Thus, at a minimum, a sequence somewhere between −19 and −13 has a function separate from TIF binding. Since the first 3′ deletion inactive in preinitiation complex formation is 3′dl −27, this start site proximal region not involved in TIF binding has a maximal size of −26 to +8.

A second region of the promoter (motif B) is required for the formation of stable preinitiation complexes. Templates that contain this motif and core motif A (see below) form preinitiation complexes that are stable for at least 1 hr, even when competing wild-type templates are added after complex formation. Motif B is not needed for transcription *per se* (and thus is not part of the core promoter), because deletions missing only motif B are fully active transcriptionally. Motif B is defined by the two functional tests described above: 5′ deletions to −48 are able to sequester transcription factors into a stable complex while deletions to −40 or beyond cannot. Deletions to −32 are transcriptionally active while those to −26 or beyond are not. Thus, after deletion of the first nucleotide pair that prevents stable binding, an additional 10 nucleotide pairs or more can be deleted without affecting transcription.

The third sequence region (core motif A) is defined indirectly. In competition prebinding experiments, the first DNA sequesters all of the TIF(s) needed for faithful initia-

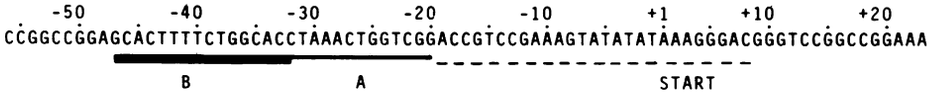

FIG. 6. Antisense strand sequence of the ribosomal RNA gene of *Acanthamoeba* in the region of the promoter. Start site motif is underlined with a dashed line, core motif A with a thin underline, and motif B with a heavy underline. The first base coding for rightward transcript is +1.

tion so that a template subsequently added to the reaction is not correctly transcribed even though excess RNAP-I is present (e.g., see Fig. 4, lanes 2–4, band B). We have shown previously that the polymerase alone is incapable of specific initiation (11). We conclude that at least one additional protein must interact with the DNA template in a sequence-specific manner to direct the polymerase to the correct start site. In templates that have motif B deleted (e.g., 5′dl −40, −37, −35, −32; Fig. 4, lanes 5–8, band A), correct initiation still occurs, so this putative interaction site (core motif A) must be present and must be functionally distinct from motif B. Core motif A can be distinguished from the start motif because 3′ deletions, which inactivate the template for transcription (3′dl −13, −16, −19), retain the ability to sequester the TIF, which interacts with motifs A and B (Fig. 3, lanes 4–6, band B). Related to this, it is also noteworthy that motif B cannot stably bind TIF(s) because templates containing motif B, but deleted in motif A, are inactive in competition prebinding. The borders of motif A are poorly defined, but they must lie somewhere between −31 and −20.

For a stable preinitiation complex to form, both core motif A and motif B must be present. This finding suggests two alternative mechanisms for stable complex formation: (*i*) a single TIF could interact with both regions, or (*ii*) more than one TIF may be present and distinct TIFs could bind to each of the motifs and interact with each other to form a stable complex. According to the first mechanism, a single protein could have two domains that interact with the template: one with core motif A and the other with motif B. Both of these interactions would contribute to the binding strength of the protein to the template. Deletion of either core motif A or B would weaken the binding, but would not eliminate it completely. The observation that deletion of motif B prevents stable complex formation but allows faithful transcription is compatible with this idea. Unfortunately, we have not been able to test the effect on transcription of core motif A deletion because 3′ deletions extending into core motif A have also deleted the required start motif and, therefore, are transcriptionally inactive.

The two motifs could also bind distinct TIFs. One of these, the one that binds to core motif A, would be sufficient to direct faithful initiation of transcription. However, this complex would be unstable and readily exchange TIF with other templates. Binding of the second TIF to motif B would stabilize the preinitiation complex so that exchange between promoters would be inhibited. Because of the lack of sequence homology between the two binding sites, we reject the variation of this mechanism in which two molecules of the same TIF would cooperatively bind to core motif A and motif B.

To date, no publications presenting direct evidence for more than one transcription initiation factor in RNAP-I systems have appeared. *In vitro* transcription is inhibited by high template concentrations (>40 μg/ml) (11), and this result has been cited as evidence for more than one TIF (15). The idea is that inhibition results from dispersal of multiple TIFs to separate DNA template molecules so that no single promoter has the correct complement of TIFs for preinitiation complex formation. We find that DNA molecules containing only one of the motifs are not capable of stably binding TIF. Both motifs must be present for formation of a stable preinitiation complex. Deletions from the 5′ end containing the region downstream of −26 (i.e., deleted in motif B and core motif A but containing the remainder of the core promoter) also do not sequester TIFs. For the above mechanism of inhibition to work, at least two of the three motifs must stably bind a distinct TIF. Our results, therefore, argue against the validity of this explanation for the inhibition by high DNA concentrations observed in the *Acanthamoeba in vitro* transcription system.

We have used TIF preparations that are essentially free of endogenous RNAP-I activity and highly purified RNAP-I incapable of faithful initiation *in vitro*. This has allowed us to determine that RNAP-I is not a part of the initial complex formed between the DNA and transcriptional proteins, a possibility not excluded by previous experiments in polymerase I systems (6–8). This result also eliminates the formal possibility that the TIF must obligatorily bind first to the RNAP-I before interacting with the template. We cannot eliminate the possibility, however, that this TIF could bind to RNAP-I before binding to the template as an alternative, and minor, kinetic route to formation of the initiation complex. It is also conceivable that this could be the major route in some organisms. In *Acanthamoeba*, the absence of motif B hinders the major route to initiation complex formation by destabilizing the preinitiation complex. With motif B-deleted mutants, the possible secondary kinetic mechanism could allow the observed diminished level of transcription.

The demonstration that the TIF(s) are capable of forming a stable preinitiation complex in the absence of RNA polymerase suggests that the preinitiation complex could remain after RNA polymerase has translocated away from the promoter region as a result of RNA synthesis. This idea is further supported by the finding that once a preinitiation complex has been formed on a given DNA template, secondary DNA templates are not transcribed even when RNA is actively transcribed from the first template (e.g., Fig. 2*C*; see refs. 6 and 7). If TIF were released upon initiation, it could bind to the secondary template, which would then be transcribed by the excess RNAP-I present in the *Acanthamoeba in vitro* system.

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