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A stable transcription complex directs mouse ribosomal RNA synthesis by RNA polymerase I

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ABSTRACT

Ribosomal RNA is synthesized from template molecules that are activated by a stable association with essential transcription factors. This activated template assembles prior to the onset of transcription as a preinitiation complex and factors remain firmly attached during active elongation as well. Sequential addition of differently marked rRNA genes to an S-100 mouse cell extract shows that the DNA binding factors of the stable complex are present in limiting quantities. They associate rapidly with template molecules and the resultant transcription complex remains intact over prolonged periods of incubation in the presence of competitor DNA. The resistance of the stable complex to the usual inhibitory effect of high DNA concentration suggests that more than one DNA binding factor recognizes the rDNA promoter region and is needed to direct faithful transcription. Finally, although the stable complex is specific for the rRNA initiation region, added vector sequences can neutralize nonspecific DNA binding components that are also present in the cell extract. This lowers the requirement for rDNA template and demonstrates that each activated rRNA gene can direct at least 10 rounds of elongation and reinitiation.

INTRODUCTION

With the recent advent of manipulable in vitro transcription systems, many significant advances have been made toward understanding eukaryotic transcription. Unlike the case in prokaryotes, the three eukaryotic RNA polymerase classes can initiate accurately in crude cell extracts but not in the purified form (1-10). Certain transcription factors thus act to supplement each class of polymerase in directing template specificity. However, the identity and mechanism of action of these factors remains largely unknown.

Studies on transcription catalyzed by RNA polymerase III have progressed the most rapidly. A purified initiation factor has been shown to interact directly with the nucleotide sequences identified earlier as the intragenic control region of 5S RNA genes (4,11,12,13). Two additional factors are required for specific transcription of 5S and tRNA genes as well

(4,5). Analogous studies in polymerase II systems have resulted in the partial purification of at least three transcription factors (7,14,15), each of which plays an undefined but essential role in directing accurate synthesis of hnRNA. However, far less is known about the transcriptional machinery associated with polymerase I. The template regions required for initiation by this polymerase have recently been elucidated (16-22), and initial titration and fractionation studies indicate that at least three factors are required for accurate rRNA synthesis (9,23).

Transcription by RNA polymerase III has been shown to occur on template molecules that are stably activated for synthesis by association with one or more factors (24,25). More recently, stable transcription complexes were also found to act as template for RNA polymerase II (26). The identification of such complexes is valuable for understanding the mechanisms of transcriptional initiation and gene activation; in addition, their isolation could greatly facilitate the characterization of transcription factors.

In this study, we demonstrate that stable complexes mediate the activation and transcription of mouse rRNA genes by RNA polymerase I in vitro. These complexes form rapidly, even in the absence of transcription, remain stable over extended periods of time and are specific for the rRNA initiation region. We also show that multiple rounds of synthesis can be directed by each transcription complex. While this manuscript was in review, a study reaching similar conclusions on polymerase I transcription complexes was published (27).

MATERIALS AND METHODS

S-100 extracts were prepared from logarithmically growing mouse tissue culture cells (L1210) and stored as previously described (1). Transcription reactions (25 µl) containing 7 µl of extract were made to 15 mM Hepes (pH 7.5), 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 90 mM KCL, 5 mM $MgCl_2$ and 300 µg/ml α-amanitin. Reactions also contained 10 µg/ml template DNA, 500 µM ATP, UTP and GTP and 50 µM α-^{32}P-CTP (2.5 Ci/mM). The template is a mouse rDNA clone (p5'Sal-Pvu; 1) truncated with either Sma I or Pvu II (Figure 1A). To allow stable complex formation, reactions were first incubated at 30°C for 10 minutes with 10 µg/ml template DNA in the presence or absence of unlabeled ribonucleotide triphosphates (rXTPs). Competitor DNA (10 µg/ml) and radioactive nucleotides were then added in 2 µl and incubation proceeded for an additional 45 minutes at 30°C. To terminate reactions, 185 µl of a

solution containing 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 0.3 M NaOAc, 0.5% SDS, 60 µg/ml tRNA and 6 mM EDTA was added. Nucleic acids were then phenol extracted, ethanol precipitated, resuspended in 4 µl of 95% deionized formamide, 0.05% xylene cyanol and 0.05% bromophenol blue and heated at 100°C for 2 minutes. Transcripts were resolved on a 4% polyacrylamide, 9 M urea sequencing gel (28) and visualized by autoradiography.

Transcripts from closed circular templates were analysed by S1 nuclease mapping as previously described (1). The probe used for these studies was a mouse ribosomal DNA clone (p5'Sal-Pvu) cleaved with Sma I at nucleotide +155, ^{32}P labeled at the 5' termini and made single stranded by Exo III treatment. RNA prepared from unlabeled transcription reactions was hybridzed in probe excess to ~8 times the $Cot_{1/2}$ and treated with S1 nuclease; the protected fragments were electrophoretically resolved and visualized by autoradiography.

RESULTS

Assembly of Stable Transcription Complexes

We have previously demonstrated that cloned mouse rRNA genes are accurately transcribed in vitro using an S-100 extract of mouse tissue culture cells (1). To investigate whether stable transcription complexes are responsible for this synthesis, we allowed differently marked rRNA genes to compete for the limiting transcription factors present in the extract. The DNA template used in these studies is a cloned mouse rRNA initiation region (p5'Sal-Pvu) that was truncated either at nucleotide +155 with Sma I or at nucleotide +300 with Pvu II (Figure 1A). Both of these run-off templates contain all the sequences that promote transcription in vitro (22). For convenience, they shall be designated as 'p155' and 'p300', according to the characteristic length of transcripts produced (Figure 1B, lanes a-d). The ability of these two templates to compete for transcription factors in vitro is demonstrated in Figure 1B. When equal amounts of p155 and p300 are simultaneously added to the reaction, equimolar quantities of the two run-off transcripts are produced (lane e). However, if p155 is preincubated in a transcription reaction for 10 minutes prior to the addition of p300 competitor, only the 155 nucleotide long RNA is produced (lane f). [To detect only those RNAs transcribed when both templates are present, the labelled nucleotide was added along with the competitor DNA.] In the reciprocal sequential addition, where p300 is added first, transcription from p155 introduced ten minutes later is completely

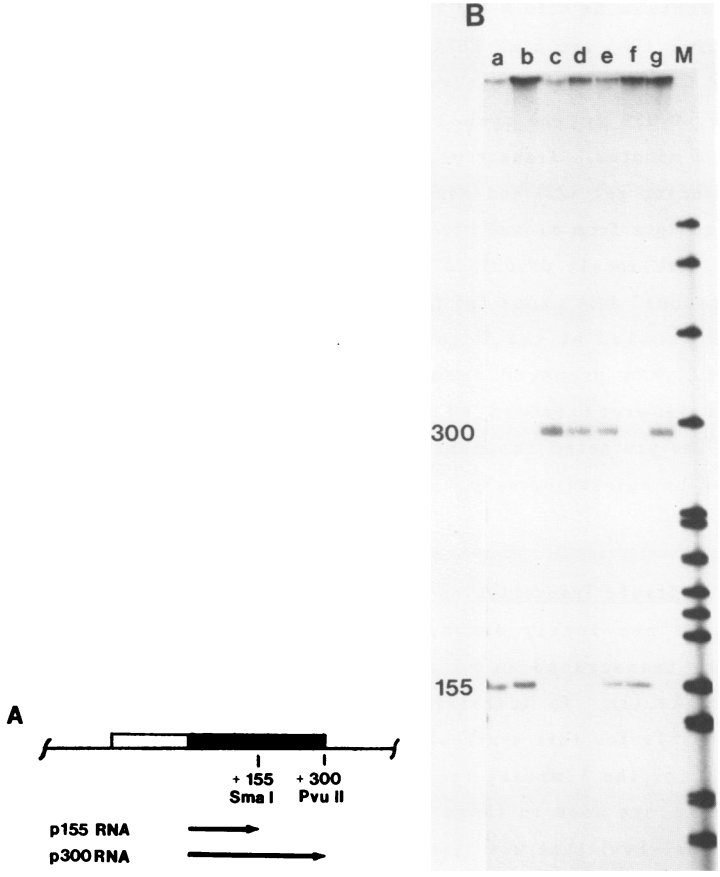

Figure 1: Run-off transcription and sequential addition studies

A: The cloned rDNA template (p5'Sal-Pvu) and relevant restriction endonuclease cleavage sites are shown above; ■ - transcribed rDNA region (nucleotide +1 to +300); □ - nontranscribed rDNA region (nucleotide -170 to +1); —— - pBR322 vector. The run-off RNA transcribed from template cleaved with Sma I at +155 (p155) or with Pvu II at +300 (p300) is depicted below.

B: Transcription reactions were allowed to preincubate with 10 µg/ml of the first DNA for 10 minutes in the absence of rXTPs. Then 10 µg/ml of the second DNA, α-^{32}P-CTP and rXTPs were added and transcription was terminated 45 minutes later: (lane a) 1st-p155, 2nd-no additions; (lane b) 1st-p155, 2nd-p155; (lane c) 1st-p300, 2nd-no additions; (lane d) 1st-p300, 2nd-p300; (lane e) 1st-p155 and p300, 2nd-nothing; (lane f) 1st-p155, 2nd-p300; (lane g) 1st-p300, 2nd-p155. An electrophoretic analysis of the purified RNA is shown. (M) is Hpa II cleaved pBR322 marker.

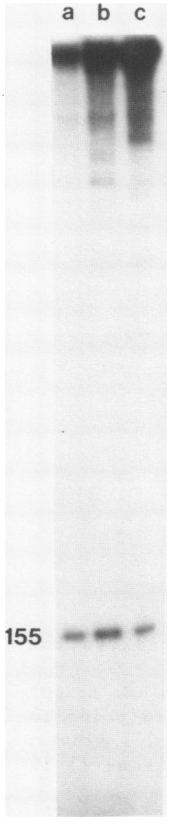

Figure 2: Preincubation with DNA lacking the rRNA promoter
Sequential addition experiments were set up as in Figure 1B: (lane a) 1st-p155, 2nd-no additions; (lane b) 1st-pBR322, 2nd-p155; (lane c) 1st-a mouse rDNA clone spanning from nucleotide +300 to +3000 and thus lacking the initiation site, 2nd-p155. High molecular weight RNAs at the top of the gel result from nonspecific end-to-end transcription. Similar results to lanes b and c are obtained when calf thymus DNA is added first and p155 second.

suppressed (lane g). The same suppression of synthesis of subsequently added p155 competitor is observed when the transcription reaction is first preincubated with closed circular rDNA. Thus, a stable transcription complex is formed on the template added first; the essential factors involved must be present in limiting amounts and are not transferable to other rDNAs subsequently added.

We have previously shown that omitting rXTPs from the in vitro reaction prevents transcriptional initiation (29). Yet results identical to Figure 1 are obtained whether or not exogenous rXTPs are present during the preincubation. This demonstrates that a 'preinitiation complex' assembles on the template in the absence of RNA synthesis. However, since this association can also form and persist in the presence of rXTPs, an 'elongation complex' is stably bound during transcription as well.

In contrast to the experiment of Figure 1, when the cell extract is

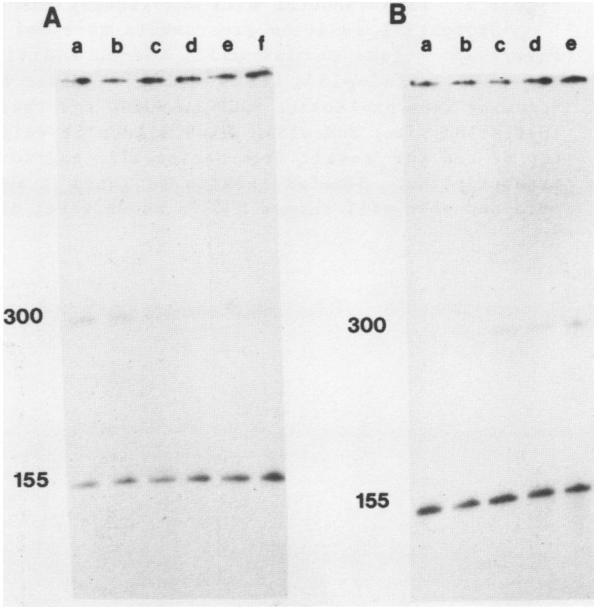

Figure 3: Time course for formation and stability of the preinitiation complex

A: Sequential addition experiments were set up as in Figure 1B, except that the time of preincubation with the p155 first DNA was varied prior to addition of the p300 competitor DNA: (lane a) 30 seconds; (lane b) 1 minute; (lane c) 2 minutes; (lane d) 5 minutes; (lane e) 10 minutes; (lane f) 15 minutes.

B: Sequential addition experiments were preincubated for 10 minutes with p155, followed by the addition of p300 (but no rXTP). After the indicated times of further incubation, a 45 minute transcription was initiated by addition of rXTPs and α-^{32}P-CTP: (lane a) 0 minutes; (lane b) 15 minutes; (lane c) 30 minutes; (lane d) 45 minutes; (lane e) 1 1/2 hours.

first preincubated with vector DNA, rDNA that lacks the initiation site or bulk calf thymus DNA, there is no suppression of transcription from the p155 template added 10 minutes later (Figure 2). Thus, formation of the stable complex is specific for template sequences surrounding the rRNA initiation site. The essential factors either do not bind to the other DNA sequences or they do so in a readily reversible manner.

Time Course for Complex Formation and Stability

To study the kinetics of complex assembly, p155 was preincubated with cell extract for various times prior to the addition of competitor p300. As shown in Figure 3A, the complex assembles very rapidly; there is preferential transcription of the first DNA after only a 30 second

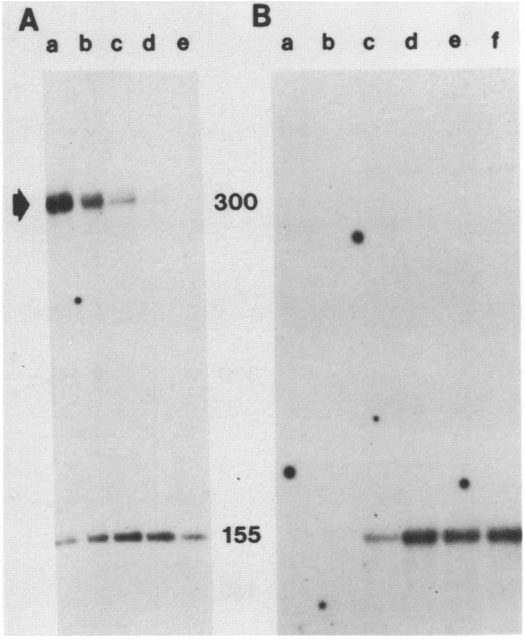

Figure 4: DNA concentration requirement for complex formation

A: Sequential addition experiments were set up as in Figure 1B, except that the amount of p155 first DNA was varied as indicated. To all reactions 10 µg/ml of p300 competitor was added 10 minutes later: (lane a) 1 µg/ml; (lane b) 2 µg/ml; (lane c) 5 µg/ml; (lane d) 7 1/2 µg/ml; (lane e) 10 µg/ml. [The apparent decrease in signal in lane e is not reproducible.]

B: An analagous experiment was set up using the indicated amounts of p155 template but no competitor DNA: (lane a) 1 µg/ml; (lane b) 2 µg/ml; (lane c) 5 µg/ml; (lane d) 7 1/2 µg/ml; (lane e) 10 µg/ml; (lane f) 15 µg/ml.

preincubation, and within 5 minutes complex formation is complete.

Once the preinitiation complex is formed, the factors remain stably bound during prolonged incubation. This was demonstrated as follows: After preincubation with p155, competitor p300 was added and the reactions were incubated for various lengths of time before initiating transcription with rXTPs. Even after a 1 1/2 hour incubation, subsequent transcription is almost exclusively from the first template (Figure 3B). A small amount of the 300 nucleotide long transcript does appear with time, but this constitutes less than 5% of the total synthetic capacity and may represent a very slow rate of complex disassembly. Thus, over extended periods of preincubation virtually all of the transcription complexes remain stably

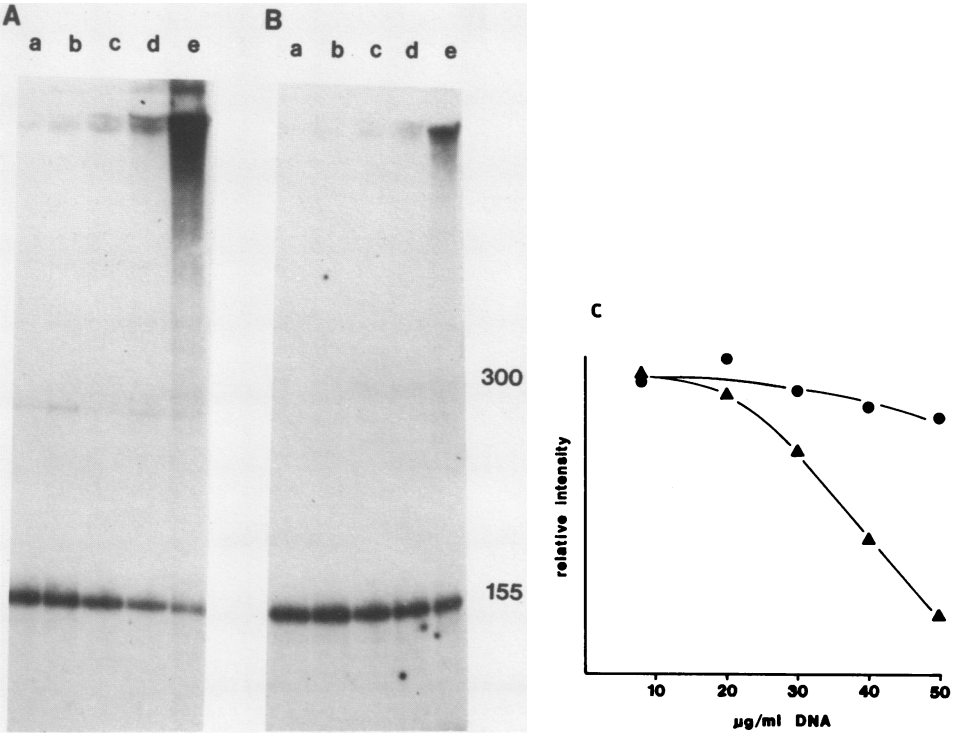

Figure 5: Complex formation prevents transcriptional suppression at high DNA concentration

A: Transcription reactions were set up with the following concentrations of p155 DNA: (lane a) 10 µg/ml; (lane b) 15 µg/ml; (lane c) 20 µg/ml; (lane d) 30 µg/ml; (lane e) 50 µg/ml.

B: Sequential additions were set up as in Figure 1B, using 10 µg/ml p155 first DNA, but adding p300 competitor to the indicated total DNA concentration, equaling that of part A: (lane a) 10 µg/ml, i.e., no addition; (lane b) 15 µg/ml; (lane c) 20 µg/ml; (lane d) 30 µg/ml; (lane e) 50 µg/ml.

C: The bands of the gels in parts A (●) and B (▲) were quantitated by densitometry.

bound to the template on which they originally formed.

DNA Concentration Requirements for rRNA Transcription and Complex Assembly

The template concentration needed for complete complex formation was determined by titrating the amount of preincubated p155 required to suppress synthesis from subsequently added p300 competitor. In the experiment shown in Figure 4A, approximately 7 1/2 µg/ml of p155 is needed to saturate the limiting, nontransferable factors and thereby prevent transcription of p300. Although this saturating value varies slightly between different extracts,

it always coincides with the template concentration needed for maximal rRNA synthesis in reactions without competitor (Figure 4B). Therefore, the amount of stable complex assembly directly correlates with the level of rRNA synthesis.

At the other end of the concentration range, increasing the template to 50 µg/ml appears to exceed the capacity of the transcription factors, for there is a marked reduction in rRNA production (Figure 5A). A similar suppression of synthesis at elevated template concentrations has been observed in other systems (3,9) and attributed to a partitioning of the different essential transcription factors onto separate DNA molecules (9,24). If this explanation for the decreased synthesis is correct, then pre-formed stable complexes should not be affected by subsequent addition of excess template, for the factors will remain bound to the first DNA. The experiment of Figure 5B-C shows that this prediction is correct: Complexes pre-formed at the optimal 10 µg/ml of p155 exhibit virtually no transcriptional inhibition when the DNA concentration is raised to 50 µg/ml with competitor p300. The magnitude of the transcriptional signal at high DNA concentration therefore depends on the history of the reaction; only if factors are first allowed to assemble into intact complexes are they resistant to partitioning onto the additional DNA molecules.

Stable Transcription Complexes Undergo Multiple Rounds of Reinitiation

The precipitous decrease in transcriptional signal below 5-7 µg/ml of template DNA (Figures 4B) as well as a plateau in synthesis at high concentrations of extract (data not shown) suggests that our cell extract also contains DNA binding factors that can limit template availability. In an attempt to maximize the synthesis per input rDNA molecule, we transcribed low amounts of template in the presence of 10 µg/ml vector DNA which might absorb such putative nonspecific DNA binding proteins. As shown in Figure 6B, this treatment permits high levels of transcription at rDNA concentrations as low as 0.25 µg/ml. This constitutes a ~10 reduction of the normal template requirement for this extract (Figure 6A). From the known specific activity of the RNA and quantitation of the autoradiographic bands, we calculate that ~1 run-off transcript was synthesized per input template molecule (see legend to Figure 6). This value can be further raised to about 10 RNAs per DNA molecule when closed circular templates are used: S_1 nuclease analysis of transcripts made at reduced DNA concentrations (where synthesis per template is maximized) shows that the stable complexes on closed circular rDNA molecules direct 10 fold more initiation events than

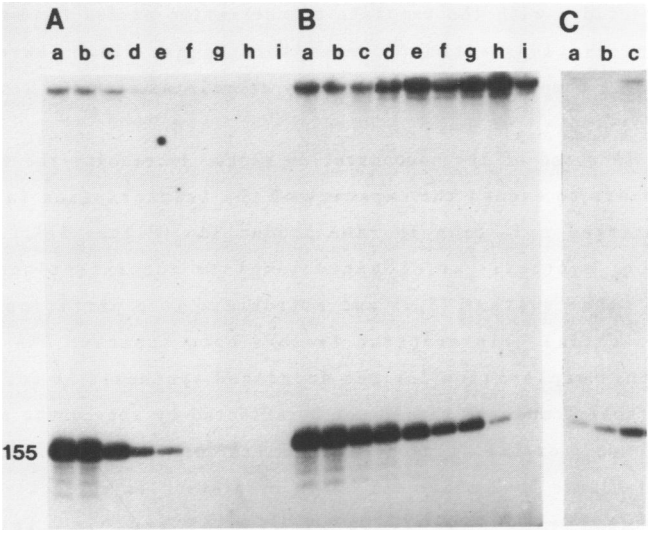

Figure 6: Addition of vector sequences raise the rRNA : rDNA ratio

A: Transcription reactions were preincubated with the indicated amounts of p155 template (in µg/ml), but no competitor DNA was added: (lane a) 7.5; (lane b) 5.0; (lane c) 2.5; (lane d) 1.25; (lane e) 0.75; (lane f) 0.5; (lane g) 0.25; (lane h) 0.125; (lane i) 0.075.

B: Identical reactions were set up as in part A except that 10 µg/ml linearized pBR322 DNA was added with rXTPs after the 10 minute preincubation.

C: Closed circular and linear templates were transcribed in unlabelled reactions and the resultant RNA was quantitated by S1 nuclease analysis: (lane a) no template added; (lane b) 0.25 µg/ml linear template; (lane c) 0.25 µg/ml closed circular templates. Lane a represents the endogenous rRNA in the cell extract that must be subtracted from the signals of lanes b and c in order to determine the amount of rRNA transcribed in vitro.

For quantitation of this experiment, scintillation spectroscopy demonstrated that the input 5µCi/1250 pmol CTP corresponds to $8x10^3$ cpm/pmole CTP. The autoradiographic standard for the experiment of 6A was a end labelled DNA fragment that was quantitated by scintillation spectroscopy, and then subjected to electrophoresis and autoradiography in parallel with run-off transcripts. By this analysis, lane g corresponds to 1300 cpm, or 0.16 pmoles of CTP, or 0.004 pmoles of the 155 nucleotide RNA; it derives from 0.003 pmoles of template rDNA. Densitometer scanning of the bands of part C demonstrate that 10-11 times as much rRNA is made from closed circular as linear templates.

those on linearized templates. We therefore conclude that the transcription complex can remain stably bound to the template through at least 10 rounds of initiation and elongation. This calculation, in fact, leads to a minimal estimate of the extent of reinitiation, for not all the genes may be engaged in transcription.

DISCUSSION

In this paper, we describe a stable RNA polymerase I transcription complex which assembles as a prerequisite to mouse ribosomal RNA synthesis. When rRNA genes are incubated with extracts prepared from growing cells, DNA molecules are rapidly assembled into active templates by interaction with one or more transcription factors. This nucleoprotein complex is indeed a stable association as indicated by its resistance to competitor DNA: When two rDNA templates are sequentially added to the in vitro reaction, complex formation on the first DNA completely suppresses transcription from the subsequently added competitor (Figure 1). Such transcriptional preference is observed even when the competitor is present in a vast molar excess (Figure 5). The activating factors associate rapidly with the template and remain bound to these same DNA molecules during prolonged incubation with competitor DNA and through multiple rounds of transcription (Figures 3 and 6). Ribosomal RNA gene activation is indeed due to complex formation and not to covalent modification of the template for factors can be released at elevated salt concentrations and will then productively rebind to other templates upon reduction of the ionic strength (Tower, J. and Sollner-Webb, B., unpublished observations). Thus one or more factors of the complex stably binds to the template and is responsible for maintaining the rDNA in a transcriptionally active state.

Assembly of a stable complex does not require ribonucleotide triphosphates, and it therefore occurs prior to the onset of RNA synthesis forming a 'preinitiation complex'. Since these same activated template molecules are selectively transcribed upon addition of rXTPs, essential factors must also remain associated with the template during active synthesis. Therefore, a stable 'elongation complex' is responsible for directing transcription. In other experiments (30), we have found that all the transcription factors are bound in the preinitiation complex but only a subset of these factors remain stably associated with the actively transcribing complex.

The data of Figure 2 demonstrates that DNA molecules lacking the rRNA initiation region do not compete with the active templates for essential transcription factors. Therefore, stable complexes have a high specificity for the rDNA initiation region. In fact, studies currently underway with various deletion mutants suggest that precisely the rDNA promoter region is required for stable complex formation (Cizewski, V., Miller, K. and Sollner-Webb, B., unpublished observations). Overall, these data demonstrate that

the polymerase I transcription complex described in this paper cannot be attributed to nonspecific events. For instance, one could propose that our cell extract contains an excess of transcriptionally inhibitory factors and a limited amount of a nonspecific anti-inhibitor (31). While such models would account for the preferential transcription of the first added DNA, they are invalidated by the specificity for the rDNA promoter region (Figure 2).

In our in vitro system, a minimal DNA concentration of ~7 1/2 µg/ml is required both for rRNA synthesis (Figure 4B) and for saturating the nontransferable transcription factors (Figure 4A), reflecting the importance of complex formation in the synthetic process. However, the minimal template requirement can be dramatically reduced when the transcription reaction is supplemented with vector DNA (Figure 6). These nonspecific sequences apparently absorb DNA binding proteins of the cell extract that otherwise reduce the effective concentration of available template molecules. Thus, only a small proportion of input DNA actually serves to direct transcription while the remainder is evidently needed to absorb nonspecific DNA binding components (32).

The relative order of vector and rDNA addition strongly affects the amount of transcription obtained at the low template concentrations. Synthesis is maximally stimulated when extract was first preincubated with rDNA (Figure 6), suggesting that transcription complexes remain stably bound to the ribosomal template while nonspecific components transfer to the subsequently added DNA. In the reverse case, where extract is first preincubated with the nonspecific DNA, there is only minimal stimulation of transcription from the small amount of ribosomal template relative to that obtained without the nonspecific DNA (data not shown). Transcription factors presumably bind to the vector in a transient manner thereby slowing their search for, and transfer to, the very small amount of subsequently added rDNA. Laboratories investigating transcription by RNA polymerase II have similarly found that adding nonspecific DNAs siginificantly lowers the template requirement for in vitro transcription (26,33). Poly I-poly C was highly effective in these studies, but in our system, it does little to stimulate transcription by RNA polymerase I (data not shown). Thus, different DNA sequences efficiently reduce the template requirement in different transcription systems; this presumably depends on the relative affinities of template and nonspecific sequences for the transcription factors and the other DNA binding components.

The synthetic capacity of our in vitro system is reduced at high DNA concentrations (Figure 5A). This is due to a suppression of stable complex formation, for raising the template concentration in reactions with preformed complexes has no inhibitory effect (Figure 5B). Activation of rRNA templates evidently requires multiple factors which must interact with the promoter region in concert. In the presence of excess DNA, these limiting factors partition nonproductively onto different template molecules unless they are first allowed to assemble into stable transcription complexes. Similarly, transcription of 5S RNA genes by polymerase III is also reduced at high DNA concentrations unless complexes are first formed with optimal amounts of template (24).

Finally, we present evidence that polymerase I stable complexes direct multiple rounds of transcriptional initiation and elongation. By lowering the template requirement with vector DNA, at least 10 transcripts can be synthesized per rDNA molecule (Figure 6). This may in fact be an underestimation of the number of reinitiation events for not all the input template molecules may be engaged in transcription. Corroborating this conclusion of multiple reiniation events, when the salt concentration of an ongoing transcription reaction is raised to or above 180 mM, production of the 155 nucleotide run-off RNA abruptly ceases (Cizewski, V. and Sollner-Webb, B., unpublished observations). Since these salt conditions should allow elongation of nascent RNA chains while preventing further initiation by RNA polymerase I (34), this provides independent evidence that the vast majority of the in vitro rRNA synthesis is due to reinitiation.

Electron microscopic visualization of in vivo rRNA transcription demonstrates that not all the rRNA genes in somatic cells are transcriptionally active (35). However, the active genes are fully loaded with nascent transcripts and are apparently reinitiating at the maximal rate. This selective all-or-none gene activation in vivo could be readily accounted for by stable complex formation that occurs in vitro: Once a rRNA gene is activated for transcription, the essential components remain firmly bound through many rounds of synthesis. However, DNA binding inhibitors can preclude this initial productive interaction. Ribosomal RNA synthesis in vivo may similarly be regulated by the relative availability of stably binding transcription factors and inhibitors.

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31. In vivo, where histones and other nonspecific inhibitory proteins are present, activating factors may also act as sequence specific anti-inhibitors, as apparently is the case for the 5S activating factors (24).
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