MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3451–3462
0270-7306/86/103451-12$02.00/0

Vol. 6, No. 10

Factors and Nucleotide Sequences That Direct Ribosomal DNA Transcription and Their Relationship to the Stable Transcription Complex

JOHN TOWER, VALERIA CIZEWSKI CULOTTA, AND BARBARA SOLLNER-WEBB*

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 14 March 1986/Accepted 24 June 1986

We have studied the protein components and nucleic acid sequences involved in stably activating the ribosomal DNA (rDNA) template and in directing accurate transcription of mammalian rRNA genes. Two protein components are necessary to catalyze rDNA transcription, and these have been extensively purified. The first, factor D, can stably associate by itself with the rDNA promoter region and is responible for template commitment. The second component, factor C, which appears to be an activated subset of polymerase I, can stably bind to the factor D-rDNA complex but not to the rDNA in the absence of factor D. A third component which had been previously identified as a rDNA transcription factor is shown to be a RNase inhibitor. Extending our earlier observation that the ~150-base-pair mouse rDNA promoter consists of a minimal essential region (residues ~−35 to ~+9) and additional upstream stimulatory domains, we now report that each of these promoter domains acts to augment the binding of the polymerase I transcription factors. A minimum core region (residues ~−35 to ~−15) is capable of stable complex formation and of binding transcription factor D. Factor C can also bind to this D-core region complex.

The understanding of gene expression in higher eucaryotes has advanced very rapidly in the last few years, and a large part of this progress has been due to the availability of in vitro transcription systems (2, 11, 20, 24, 28, 31, 35, 36). In the case of transcription of mouse rRNA genes catalyzed by RNA polymerase I, the initiation site is known (12, 24, 37), and cloned mouse rRNA genes are accurately transcribed when incubated with an S-100 extract of mouse tissue culture cells (12, 24, 28). The rDNA region that directs this initiation has been determined by analyzing the transcriptional capacity of systematic series of 5′ and 3′ deletion mutants. Surprisingly, a small promoter region (residues ~−35 to ~+9) is sufficient under optimal transcription conditions (13, 25, 38), while the importance of increasingly larger 5′ flanking regions becomes evident when reaction conditions are made more stringent (26).

Fractionation of cell extracts by phosphocellulose chromatography has revealed three activities which appear to be necessary for the accurate transcription of mammalian rRNA genes (27). These were designated A, C, and D and represent the 0.1 M KCl flowthrough, the 0.4 to 0.6 M KCl eluant, and the 0.6 to 1.0 M KCl eluant, respectively. By cross-combining fractions derived from human and mouse S-100 extracts, fraction D was shown to contain the component responsible for the species specificity of polymerase I transcription, while A and C were relatively species nonspecific (27).

The ribosomal DNA (rDNA) molecules that serve as template in the in vitro reaction become activated for transcription by stable association with essential extract factor(s). This stable complex forms rapidly and remains intact for prolonged periods and evidently through multiple rounds of synthesis (7, 34). Stable preinitiation complexes appear to be general features of eucaryotic transcription, for they have also been reported for genes transcribed by polymerase II and III (3, 9, 18, 30). We have recently found that these stable transcription complexes can be quantitatively pelleted upon centrifugation of an in vitro transcription reaction mixture (8). The activated rDNA template that is recovered in the pellet fraction is freed from the vast majority of the extract protein, polymerase I, and transcriptionally inactive rDNA molecules, yet the pellet retains the full transcriptional activity of the parent reaction.

To better understand the mechanism and regulation of transcriptional initiation by RNA polymerase I, we have analyzed the constituents of the stable rDNA transcription complex. To this end, we have isolated the rDNA transcription factors by centrifugal fractionation, resolved them chromatographically, and determined which activities are responsible for and which are associated with the stable complex. In addition, the rDNA sequences involved in stable complex formation were identified. The entire promoter region was found to have a role in augmenting stable binding of transcription factors, while a minimal domain extending from residue ~−35 to ~−15 was found to be sufficient for complex formation due to its ability to bind transcription factor D.

MATERIALS AND METHODS

Transcription assays. S-100 extracts were prepared from logarithmically growing mouse tissue culture cells (line L1210) and HeLa cells as previously decribed (8, 24). In vitro transcriptions were performed and analyzed basically as described previously (7). The 25-μl reaction mixtures contained a final concentration of 90 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pH 7.9), 1 mM dithiothreitol, 0.1 mM EDTA, 500 μM ATP, 500 μM GTP, 500 μM UTP, 50 μM (1 to 3 μCi) [α-^{32}P]CTP, 300 μg of α-amanitin per ml, and 5 to 12 μg of

* Corresponding author.

rDNA template per ml. The mouse rDNA template was either clone p5'Sal-Pvu (24) (truncated with *Sma*I at nucleotide +155 or with *Pvu* II at nucleotide +300) or a 5' or 3' deletion mutant (26). The human rDNA template was clone pETS-RB (truncated with *Sst*I to produce a 950-nucleotide transcript) or pETS-RS(B) (truncated with *Bam*HI to produce a 700-nucleotide run off transcript), both kindly provided by Norman Arnheim (21). The enzymatic activities were contributed by 5 or 7 μl of S-100 extract, pellets and supernatants derived from single reactions, or the indicated volumes of the chromatographically derived fractions or both. Where indicated, 20 U of RNasin (Promega Biotec) was also added. Reactions involved either a 45-min incubation in the presence of ribonucleoside triphosphates (rXTPs) or preincubation in the absence of rXTPs followed by a period of synthesis in the presence of rXTPs; the durations of the incubations are indicated in the figure legends. Reactions were then terminated, and RNA was isolated, electrophoretically resolved on 4% acrylamide–9 M urea gels, and visualized by autoradiography as described previously (24). Band intensities were quantitated by densitometric scanning of autoradiograms exposed within the linear range.

RNA polymerase I was assessed for its ability to catalyze nonspecific synthesis in a reaction mixture containing excess sonicated calf thymus DNA and 1.5 mM $MnCl_2$ (8). After extraction with phenol, the resultant RNA was resolved on a 4% acrylamide–9 M urea gel, visualized by autoradiography, and quantitated by densitometry. The size distribution of each sample also provides an independent assessment that there is not a significant amount of contaminating nuclease activity. Since the incorporation is not a simple linear function of the amount of polymerase I, assays were similarly performed on a series of dilutions of selected samples to provide activity standards.

C activity was assessed by adding various amounts of the sample of interest to standard transcription reactions that instead contained either extract prepared from stationary-phase cells (32) or an excess of partly purified D in place of extract. Determinations were made in the range in which the assays were linear with the amount of added C, and virtually identical relative C activities were deduced from the two kinds of assays.

D activity was assessed by adding various amounts of the desired samples to transcription reaction mixtures that contained excess partly purified factor C in place of extract. Alternatively, D activity was determined by adding various amounts of the samples to standard mouse transcription reaction mixtures that instead contained human S-100 in place of mouse S-100 extract and thus could not transcribe mouse rDNA without added mouse D. Assessments were made under conditions where the reactions were responsive to the added D concentration. These two assays yielded similar relative D activities.

Fractionation of transcription components. To prepare the supernatant and pellet fractions, preincubated reactions were centrifuged at 10,000 × *g* for 5 min at 4°C, as described previously (8), and used without further storage. These pellets contain ~1% of the extract protein, an observation reported previously (8). To release the transcription factors from the sedimented DNA, pellets were suspended in one-sixth of the original reaction volume of a solution containing 0.1 mg of micrococcal nuclease per ml, 0.625 mM $CaCl_2$, 0.1 mM Tris (pH 7.9), and 0.1 mM EDTA and were incubated for 10 min at 30°C; nuclease digestion was terminated by the addition of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid] to 1.5 mM, and the digested pellets were then brought to transcription reaction conditions.

Resolution of the transcription components by step elution from phosphocellulose was carried out basically as described previously (27). Cell extract (~20 mg of protein per ml) was diluted with buffer I (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol), adjusted to a KCl concentration of 0.1 M, and loaded onto a phosphocellulose (P-11; Whatman) column of equal volume equilibrated with buffer I containing 0.1 M KCl; the column was washed with this buffer. Initially, the column was sequentially eluted with buffer I containing 0.4, 0.6, and 1.0 M KCl (27), but in later experiments, the B, C, and D step elutions were performed with buffer I containing 0.4, 0.8, and 1.2 M KCl, respectively. Fractions equal to 0.1 bed volume were collected, and protein concentration was determined by using Coomassie dye reagent (Bio-Rad Laboratories). The peak two or three fractions were pooled, dialyzed for ~5 h against buffer II (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$) containing 0.1 M KCl, aliquoted, and stored at −70°C.

To purify the transcription components by using DNA-dependent precipitation and gradient elution from phosphocellulose, a 30- to 50-ml mock transcription reaction mixture containing S-100 extract and 7 μg of unsheared calf thymus DNA per ml was preincubated for 45 min at 30°C in the absence of rXTPs. The factors were sedimented and released as described above, and the digested pellet was brought to 0.1 M KCl and loaded directly onto a phosphocellulose column of one-half the volume of starting cell extract. The column was eluted with a 30-ml linear gradient of 0.4 to 1.5 M KCl in buffer I, and 1-ml fractions were collected, dialyzed against buffer II containing 0.1 M KCl, aliquoted, and stored at −70°C.

For fractionation of C and D with double-stranded (ds) DNA cellulose (Sigma Chemical Co.), the column was loaded with S-100 extract diluted to 0.1 M KCl with buffer II and was step eluted with buffer II containing 0.3 M (factor C) or 1.5 M (factor D) KCl. The resultant D fraction was further purified by gradient elution from a heparin-agarose column (Bio-Rad). The column was approximately one-fifth of the volume of the starting extract and was preequilibrated with buffer II containing 0.4 M KCl; the D fraction was diluted to 0.3 M KCl with buffer II before loading. The column was then eluted with a linear gradient of 7.5 volumes of buffer II containing 0.4 to 1.0 M KCl, and the resulting fractions (~0.25 column volumes each) were dialyzed against buffer II containing 0.1 M KCl, aliquoted, and stored at −70°C. D activity eluted as a discrete peak centered at ~0.62 M KCl. The ds DNA-cellulose C activity was further purified by gradient elution from DEAE-Sephadex A25 (Sigma) followed by gradient elution from heparin-Sepharose (Pharmacia). This procedure, which is described elsewhere (32; B. Sollner-Webb et al., UCLA Symp. Mol. Cell. Biol. New Ser., in press; J. Tower and B. Sollner-Webb, manuscript in preparation), results in a C fraction in which ~50% of the protein is RNA polymerse I.

Plasmid constructs. p5'Sal-Pvu contains the rDNA sequences from residues −168 to +300 (24). Construction of the 5' deletions and the less extensive 3' deletions has been described previously (26). 3'Δ − 39 is a subclone of the rDNA region from −163 (*Eco*RI) to −39 (*Sau*3AI) of 5'Δ − 163 (26) inserted between the *EcoRI* and *Bam*HI sites of pBR322. 3'Δ − 76 and 3'Δ − 114 were constructed by subcloning the rDNA fragments of 5'Δ − 163 from −163

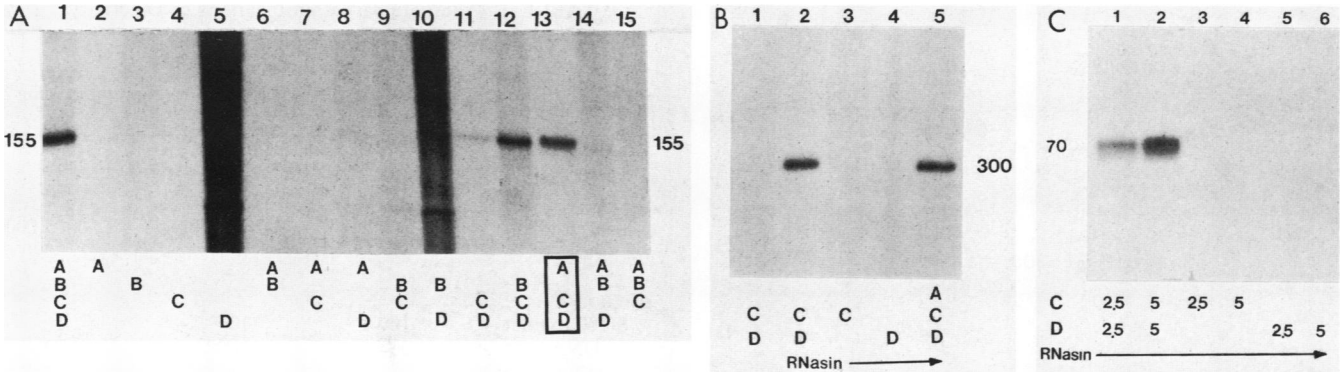

FIG. 1. Phosphocellulose fractions required for accurate transcription. (A) Mouse S-100 extract was fractionated by phophocellulose chromatography (27), and 5-μl samples of the indicated fractions were assayed for their ability to support accurate transcription of p5'Sal-Pvu, truncated to produce a 155-nucleotide runoff transcript. The reaction mixtures were incubated in the presence of rXTPs for 45 min at 30°C. These A, B, C, and D fractions contain approximately 50, 35, 10, and 5% of the starting extract protein, respectively. The nonspecific synthesis (lanes 5 and 10) is due to a combination of components in this crude D fraction and contaminating polymerase I; it is not observed with fractions prepared by using optimized KCl concentrations (panels B and C) or by gradient elution (Fig. 3B) nor with highly purified C and D (Fig. 3C and 4A). Fraction B had previously been reported by others (27) to be required to reduce background synthesis from recombined crude fractions, evidently due to poly(ADP-ribose)polymerase activity (17). (B) Phosphocellulose fractions were prepared from mouse S-100 extract by using KCl concentrations optimized to eliminate cross-contamination, as described in Materials and Methods. Samples (5 μl) of the indicated fractions were assayed for their ability to support accurate transcription from p5'Sal-Pvu, truncated to direct a 300-nucleotide runoff transcript. Where indicated, RNasin was added. Reaction mixtures were incubated as described for panel A. (C) Phosphocellulose fractions were prepared from HeLa cell S-100 extracts with the optimized KCl concentrations, and 2.5-μl (lanes 1, 3, and 5) or 5-μl (lanes 2, 4, and 6) samples of the indicated fractions were assayed for the ability to transcribe the human rDNA template pETS-RS(B) truncated with *Bam*HI to produce a 700-nucleotide runoff transcript. RNasin was added to all reaction mixtures, and incubations were as described for panel A.

(*Eco*RI) to −76 (*Hae*III partial digest) or from −163 (*Eco*RI) to −114 (*Hae*III complete digest) between the *Pvu*II and *Eco*RI sites of pBR322.

The 3' deletions +9, +2, −5, −10, and −20 were subcloned so as to also be a 5'Δ − 39. To this end, the rDNA regions extending from −39 (*Sau*3AI) to the endpoints of the 3' deletions (*Bam*HI [26]) were inserted in the clockwise orientation into the *Bam*HI site of pBR322.

For the determination of the 5' and 3' borders of the rDNA regions involved in the binding of transcription factors, each assay was repeated at least three times, and more than one template preparation was assessed. The results were highly reproducible.

RESULTS

Phosphocellulose chromatography of the S-100 extract. To study the mechanism of rRNA gene expression, we wanted to resolve and significantly purify the components that are required for transcription and for stable complex formation on mouse rDNA. To this end, the mouse S-100 extract was initially fractionated by phosphocellulose chromatography into a 0.1 M KCl flowthrough and a 0.4, 0.6, and 1.0 M KCl eluant (fractions A through D, respectively) as described by Mishima et al. (27). In basic confirmation of previous results (23, 27), accurate and efficient transcription of cloned mouse rDNA was only obtained with the combined fractions A, C, and D or the combined fractions B, C, and D (Fig. 1A, lanes 1, 12, and 13). Yet with these salt elutions, polymerase I is found in both fraction C and fraction D (23, 27) (Fig. 1A), and this fraction D is generally contaminated with low amounts of C activity. When the salt concentration of the elution buffers was instead raised to 0.4 M (B), 0.8 M (C), and 1.2 M (D) KCl, however, the D activity cleanly resolved from both the C and the bulk polymerase I activities. Assay of these fractions over a range of protein and rDNA concentrations demonstrates that specific transcription is now absolutely dependent on the presence of both fraction C and fraction D (Fig. 1B and C).

Although a strong transcriptional signal requires phosphocellulose fractions A, C, and D (or B, C, and D), a low amount of transcript was frequently observed in reaction mixtures containing fractions C and D in the absence of fraction A or B (Fig. 1A, lane 11). From a number of lines of evidence, we conclude that the putative transcription factor in fraction A (23, 27) augments RNA accumulation only by contributing a cellular ribonuclease inhibitor that allows detection of the transcription catalyzed by factors in fractions C and D. First, as shown by the instability of exogenously added RNA, fractions C and D have significant RNase activity, and this RNase activity was suppressed by components in fractions A or B (data not shown). Second, when the commercially available RNase inhibitor RNasin was added to a reaction mixture containing both fraction C and fraction D, the runoff transcript was efficiently accumulated (Fig. 1B, lane 2), and RNA added to this reaction was also stabilized by RNasin (not shown). Finally, the addition of fraction A to a reaction mixture containing C, D, and RNasin caused no further increase in the amount of resultant transcript (Fig. 1B, lane 5). Thus, the polymerase I transcription factors are contained in fractions C and D, and the stimulatory activity in fraction A is an RNase inhibitor that can be replaced by RNasin.

As with the mouse system, human S-100 extracts derived from HeLa cells can be similarly fractionated by phosphocellulose chromatography to yield equally well resolved C and D activities. These human C and D fractions support efficient transcription of human rDNA in combination with RNasin (Fig. 1C, lanes 1 and 2).

Enrichment of transcription factors by using DNA-dependent precipitation. We have previously reported a method for the rapid and selective sedimentation of stable

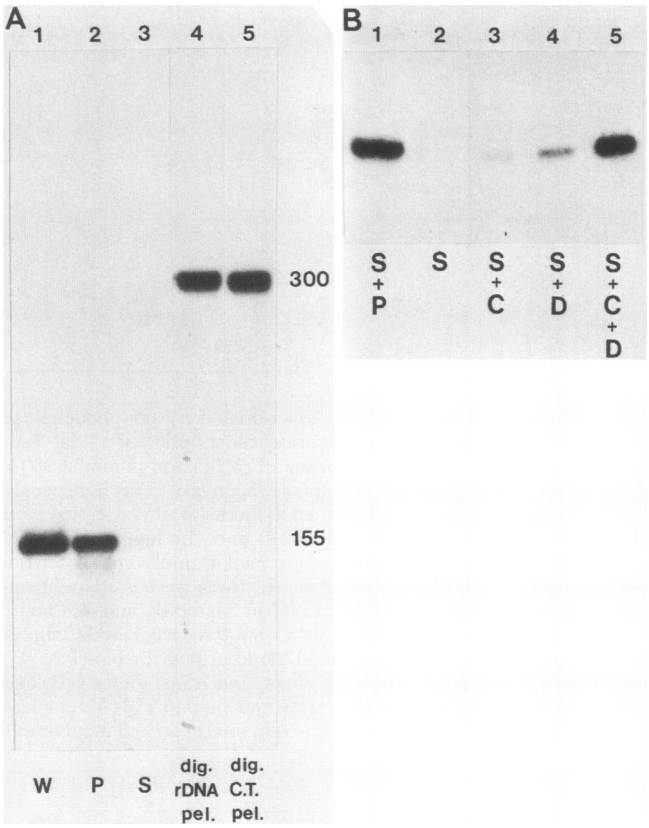

FIG. 2. Isolation of transcription factors by DNA-dependent precipitation. (A) Transcription reactions mixtures (75 μl) containing either p5'Sal-Pvu truncated to produce a 155-nucleotide transcript (lanes 1 through 4) or calf thymus DNA (lane 5) were preincubated for 45 min at 30°C in the absence of rXTPs. Reaction mixtures were then centrifuged, supernatants were removed, and pellets were suspended in transcription buffer at 4°C. Alternatively, pellets were treated with micrococcal nuclease, and the reactions were then terminated with EGTA and brought to transcription conditions. Although the transcription factors do not sediment on small fragments of nonspecific DNA (8), the large segments of calf thymus DNA afford quantitative recovery of activity. All reactions were then supplemented with p5'Sal-Pvu truncated to produce a 300-nucleotide runoff transcript and were incubated for an additional 45 min at 30°C. Finally, a 10-min period of synthesis was initiated by the addition of rXTPs. Lane 1, Whole reaction (no centrifugation or digestion); lane 2, rDNA pellet fraction (no digestion); lane 3, supernatant fraction from the pellet in lane 2; lane 4, micrococcal nuclease-digested rDNA pellet fraction; lane 5, micrococcal nuclease-digested calf thymus DNA pellet fraction. (B) Supernatant and pellet fractions were prepared from standard transcription reaction mixtures containing p5'Sal-Pvu truncated to produce a 155-nucleotide runoff transcript. The reaction mixtures were preincubated for 10 min in the absence of rXTPs and then centrifuged, and the supernatants were removed and pooled. The indicated fractions were combined, supplemented with rXTPs, and incubated at 30°C for 45 min. Additional template was added to reaction mixtures 2 through 5 to give a final concentration of ~7 μg/ml. Lane 1, Pellet suspended in supernatant; lane 2, supernatant; lane 3, supernatant fraction plus 2.5 μl of fraction C; lane 4, supernatant plus 2.5 μl of fraction D; lane 5, supernatant plus 2.5 μl of C and 2.5 μl of fraction D. A reaction mixture containing RNasin and 2.5 μl each of fractions C and D yielded an amount of transcript equal to that of lane 5. Measurement of nonspecific synthesis by using calf thymus DNA in the presence of 1.5 mM $MnCl_2$ (8; data not shown) demonstrated that the supernatant fraction contains ~90% of the RNA polymerase I activity of the parent reaction. In all of the extracts that we examined, D was in excess over C; from the data in this paper, one might anticipate that C would not pellet quantitatively when present in excess over D.

TABLE 1. Alternate partial purifications of D activity[a]

Method, fraction	Relative total activity (%)	Relative total protein (%)	Fold purification
Method 1			
S-100 extract	100	100[b]	1
Digested pellet	90–100	0.5–2	~100
P-11 gradient peak	~50	~0.01–0.02	2,500–5,000
Method 2			
S-100 extract	100	100[c]	1
ds DNA-cellulose	90–100	ND[d]	ND
Heparin-agarose peak	~50	~0.01	~5,000

[a] Some data are expressed as the range of values obtained in different experiments. Values are expressed relative to those obtained with S-100 extract.
[b] 500 mg.
[c] 2,000 mg.
[d] ND, Not determined.

preinitiation complexes (8), and we have now adapted this procedure for use as an initial step in the purification of transcription factors. Upon centrifugation of an in vitro transcription reaction mixture, ~99% of the extract protein and ~90% of the polymerase I activity remain in the supernatant (8), yet the rRNA synthetic activity is efficiently recovered in the pellet fraction (Fig. 2A, lanes 1 through 3). These centrifugally isolated transcription components are stably bound to the rDNA with which they were pelleted, for they do not transcribe added competitor template (Fig. 2A, lane 2). However, to use these pelleted factors for subsequent chromatographic fractionation, they must first be released from the DNA to which they are bound. This can be readily accomplished by treatment of the pellet with micrococcal nucleases; the factors of a nuclease-digested pellet are now able to transcribe an added competitor template at control levels (Fig. 2A, lane 4). Furthermore, to facilitate preparative-scale application of the pelleting procedure, we find that high-molecular-weight calf thymus DNA can be used in place of mouse rDNA in the precipitation step (Fig. 2A, lane 5).

To assess whether the pelleting procedure efficiently removes both the C and D activities from the soluble fraction, the supernatant was supplemented with rDNA template and either phosphocellulose-derived C (Fig. 2B, lane 3) or phosphocellulose-derived D (lane 4). These reactions yielded only a very low level of rRNA synthesis relative to either a supernatant-plus-pellet reaction (lane 1) or to a similar reaction supplemented with both fraction C and fraction D (lane 5), demonstrating efficient pelleting of both transcription factors. Indeed, since only ~1% of the extract protein partitions to the pellet (8) (Table 1), both the C and D activities are purified ~100-fold by the centrifugal isolation procedure.

The C and D activities released from the pelleted complex by micrococcal nuclease treatment were subsequently fractionated by phosphocellulose chromatography, using a linear KCl gradient. The C (Fig. 3A) and D (Fig. 3B) activities eluted as discrete and well-resolved peaks. RNA polymerase I activity coeluted with C activity (Fig. 3A). For Fig. 3B, the fractions were assayed for D activity by complementing with the fraction (no. 12) containing maximal C activity. This

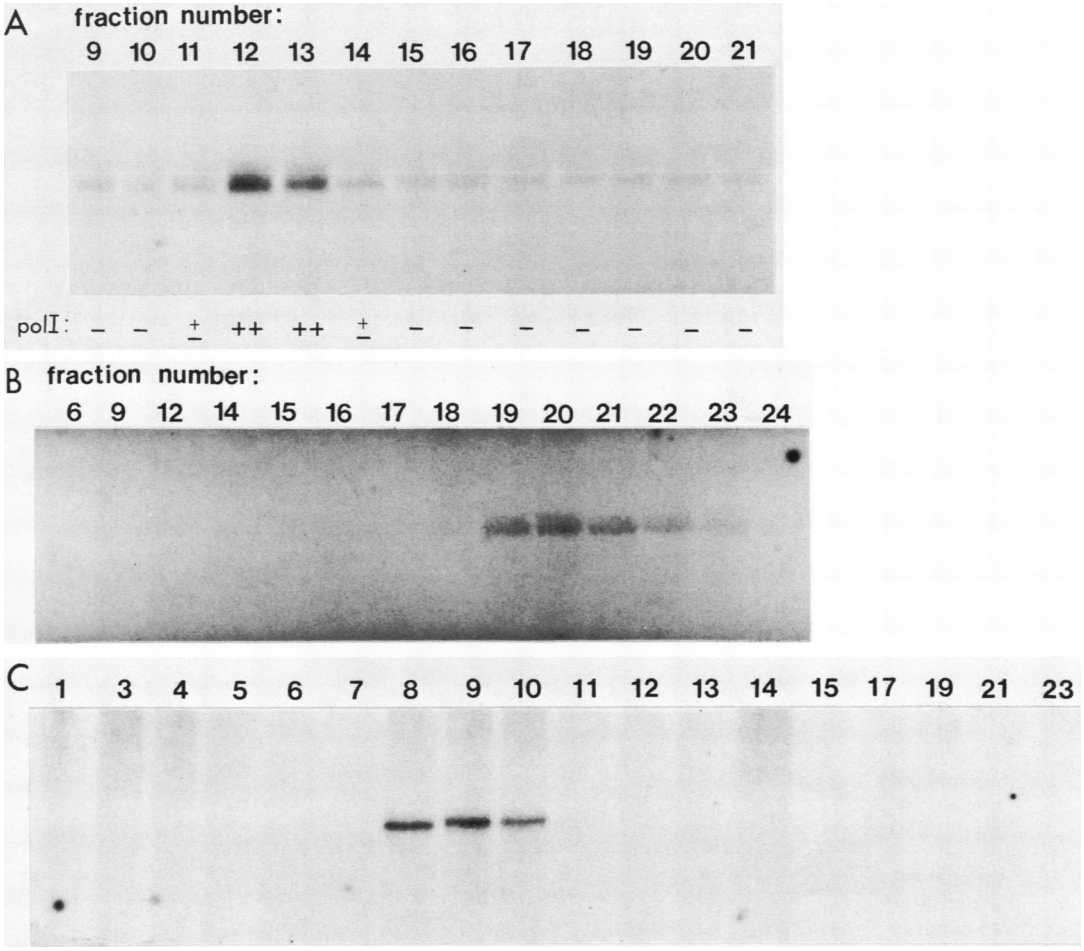

FIG. 3. Assay of gradient fractions for C and D activity. (A) Samples (7 μl) of the indicated phosphocellulose gradient fractions were assayed for C activity in a 45-min incubation at 30°C in the presence of rXTPs. The low background synthesis observed in all lanes arises because an extract of stationary-phase cells was used in this assay, an extract which we (32) and others (4) have found supports only a relatively low level of transcription unless supplemented with additional C activity. Paralleling the C activity, RNA polymerase I activity is contained in fractions 12 and 13 which elute at ~0.65 M KCl. (B) Samples (5 μl) of the indicated phosphocellulose gradient fractions were added to transcription reaction mixtures containing RNasin and 5 μl of fraction 12, the peak of C activity in Fig. 6A. The reaction mixtures were incubated in the presence of rXTPs for 45 min at 30°C. Fractions 19 through 21 eluted at ~1.0 M KCl and contributed ~0.03 μg of protein to the reactions. (C) Factor D was purified by step elution from ds DNA-cellulose followed by gradient elution from heparin-agarose, as described in Materials and Methods. Samples (7 μl) of the indicated gradient fractions were assayed for D activity in combination with 5-μl samples of heparin-Sepharose C fraction and RNasin. Reaction mixtures were preincubated with runoff template for 10 min before the initiation of a 3-min period of synthesis by the addition of rXTPs.

demonstrates that accurate transcription is catalyzed by the combination of two well-resolved activity peaks.

We have also used alternate chromatographic fractionations to obtain C and D (described in Materials and Methods), but these have not resolved either activity into multiple components. C and D activities were first separated by chromatography on ds DNA-cellulose, and the D was further purified by gradient elution from heparin-agarose (Fig. 3C). This resulted in a D preparation that, like the one illustrated in Fig. 3B, contains ~1/10,000 of the extract protein and is ~5,000-fold purified by its estimated specific activity (Table 1). The C activity from ds DNA-cellulose has been further purified by gradient elution from DEAE-Sephadex followed by gradient elution from heparin-Sepharose (32; see Materials and Methods). At each step, RNA polymerase I activity coelutes with C activity, and the resultant material is ~50% pure in the RNA polymerase I polypeptides. The combination of these resultant C and D fractions and RNasin also catalyzed efficient transcription of the mouse template Fig. 4A, lanes 9 through 11). These C and D fractions and those obtained by phosphocellulose chromatography of the digested pellet gave the same results in all of the studies described below.

Proteins of the stable complex. To determine which components are required for stable complex formation, we assayed the ability of the C and D activities to stably associate with a rDNA promoter region. In the experiment shown in Fig. 4A, mouse C and D fractions were preincubated, individually and in combination, with a mouse rDNA template truncated to produce a 528-nucleotide runoff transcript. The reaction mixtures were then supplemented with a competitor template truncated to produce a 734-nucleotide runoff transcript and the remaining fraction, and after an additional incubation period, synthesis was initiated by the addition of rXTPs. Preferential transcription of the first template indicates stable complex formation during the preincubation. Lanes 2 and 3 of Fig. 4A confirm that a stable complex was indeed formed in the presence of both fractions

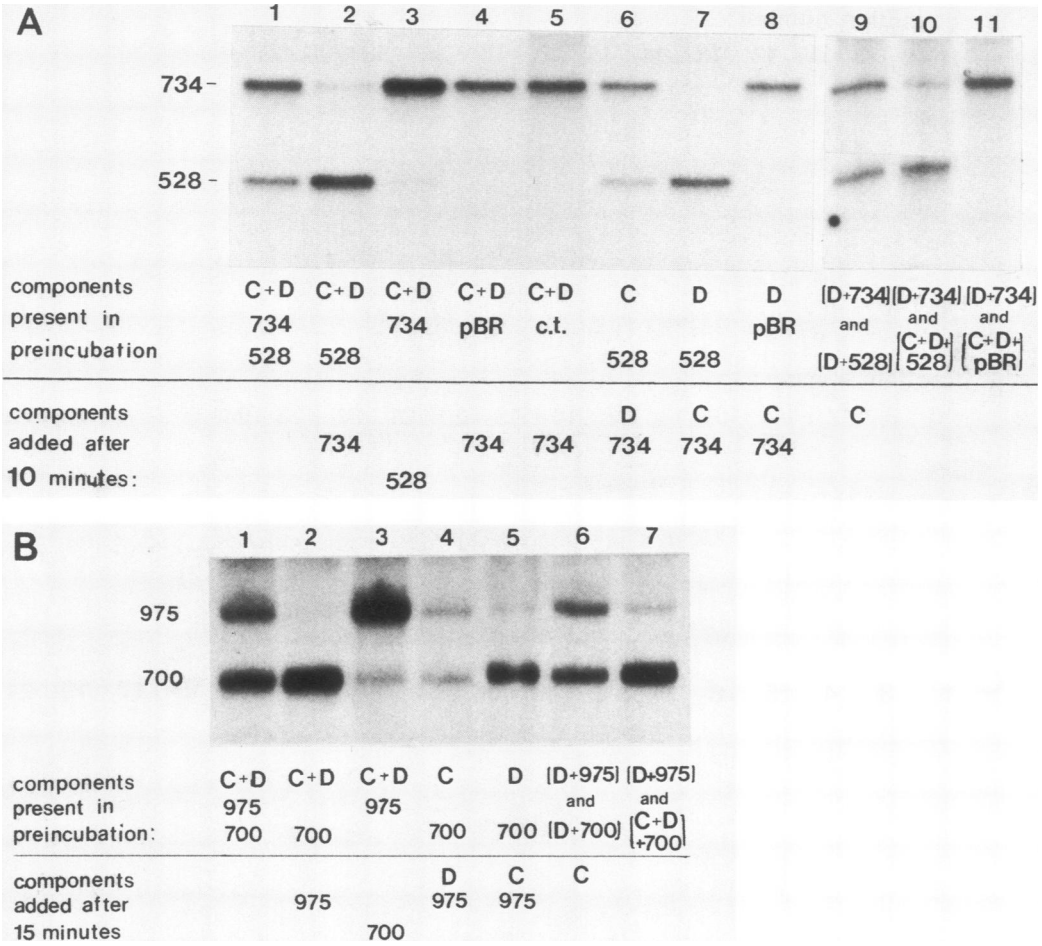

FIG. 4. Identification of the factors which comprise the stable complex. (A) Mouse cell reactions. In lanes 1-8, 4 μl of C fraction and 10 μl of D fraction were preincubated for 10 min alone and in combination with the first DNA, which encodes a 734- or 528-nucleotide runoff transcript, as indicated above the line. The reaction mixtures were then supplemented with the other components neccesary for transcription and a competitor template, as indicated below the line. After an additional 10-min incubation, rXTPs were added to initiate a 3-min period of synthesis. In lanes 9 through 11, D was preincubated for 10 min in two separate precursor reaction mixtures, each containing one of the runoff templates. These preparations were then mixed and incubated for another 10 min before the addition of rXTPs to initiate a 3-min synthesis period. In lane 9, C was added subsequent to the mixing of the D-rDNA complexes; in lane 10 and 11, C was included in the preincubation with the 528-nucleotide runoff template and the pBR322 vector, respectively. (B) Human cell reactions. Samples (5 μl each) of the human C and D fractions were preincubated alone and in combination with a 975- or 700-nucleotide human runoff template for 15 min, and the reaction mixtures were then supplemented with the other components, as indicated. After an additional 30-min incubation, rXTPs were added to initiate a 10-min period of synthesis. The mixing experiments of lanes 6 and 7 were performed analogously to those described for panel A, lanes 9 and 10.

C and D, for the first template was transcribed with a ≥20-fold preference. When only fraction D was preincubated with the 528-nucleotide runoff template, subsequent transcription was again directed almost exclusively by this rDNA (Fig. 4A, lane 7), indicating that factor D is capable of efficient stable complex formation in the absence of fraction C. This D binding is indeed specific for the rDNA promoter region, for preincubation of D with vector DNA did not significantly impede transcription of the subsequently added competitor template (Fig. 4A, lanes 4 and 5). In contrast, when only fraction C was preincubated with the first template, no preferential transcription was detected (lane 6); therefore, factor C is unable to form a stable complex in the absence of D. Other experiments (not shown) demonstrate that the amount of transcription in these reconstituted reactions is limited by the availability of C; hence, the failure to detect the stable association of C with the rDNA template truly reflects its inability to stably bind to these sequences and is not simply due to the presence of an excess of factor C.

Although factor C does not by itself stably associate with the rDNA, C is indeed a component of the stable preinitiation complex. To demonstrate this, complexes of factor D and each of the two templates were formed by preincubation in separate reaction mixtures. When these preparations were mixed before the addition of factor C, this C factor distributed approximately equally to the two factor D-template complexes, as evidenced by similar amounts of transcription from both templates (Fig. 4A, lane 9). However, when factor C was included in the preincubation with factor D and the 528-nucleotide runoff template, it did not effectively redistribute to the factor D-734-nucleotide runoff template complex, as evidenced by the preferential production of the 528-nucleotide transcript (Fig. 4A, lane 10). Thus, factor C binds stably to the factor D-rDNA complex but not to free rDNA. Moreover, this factor C binding is indeed dependent

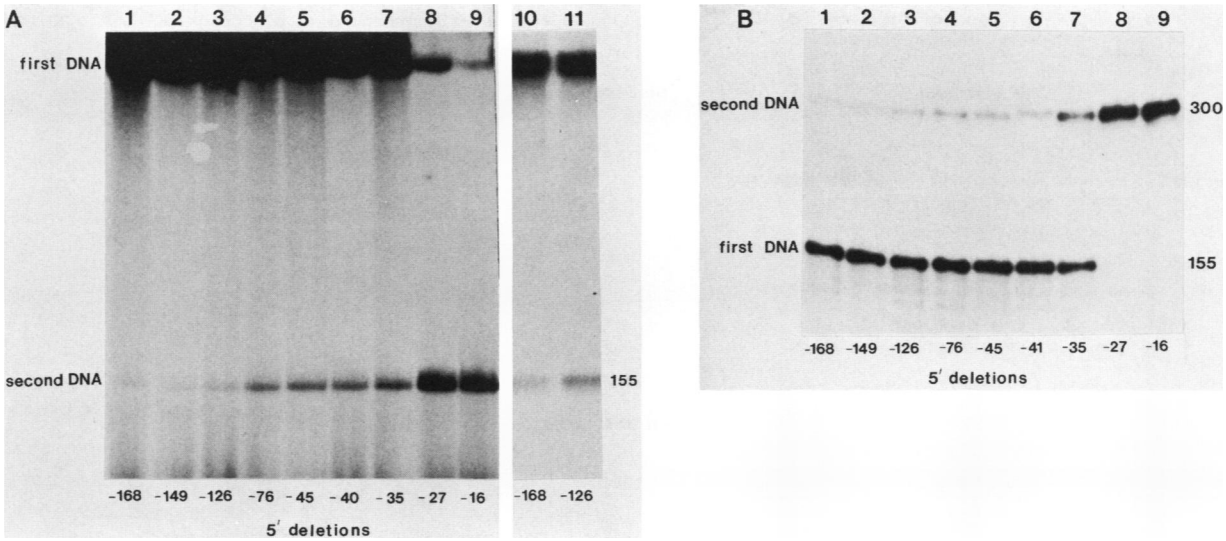

FIG. 5. 5′ borders of the rDNA region required for stable complex formation. The 5′ deletion mutants of the mouse rDNA promoter region were preincubated with mouse S-100 cell extract for 10 min at 30°C. An equimolar amount of a p5′ Sal-Pvu competitor template was then added, and a 45-min period of synthesis was initiated by the addition of rXTPs. (A) The indicated templates were closed circular, and the competitor was truncated to direct a 155-nucleotide runoff transcript. Lanes 10 and 11 are from a duplicate experiment in which a different S-100 extract and different preparations of plasmid DNA were used, and they illustrate that although stable complex formation on 5′Δ-126 is impaired to only a limited extent, the effect is quite reproducible. (B) The indicated templates were truncated to direct a 155-nucleotide runoff transcript, and the competitor template was truncated to direct a 300-nucleotide runoff transcript.

upon a factor D-promoter complex, for factor C is not sequestered by a mixture of factor D and pBR322 (Fig. 4A, lane 11).

An analogous experiment was performed with C and D fractions derived from an S-100 extract of HeLa cells and human rDNA templates truncated to produce 700- and 975-nucleotide runoff transcripts, respectively (Fig. 4B). The human cell extract fractions displayed the same properties as those from mouse. Factor D stably associated with the template by itself (Fig. 4B, lane 5), while C only bound in a stable preinitiation complex when D was also bound (lanes 4 and 7). It might be noted that the stable binding of factor D is species selective, for preincubation of mouse D with human rDNA does not impair subsequent transcription of a mouse template (see Fig. 7A, lane 9).

rDNA regions involved in stable complex formation. Extending our earlier observation that the mouse rDNA promoter consists of multiple domains that extend over a 150-bp region (26), we next determined the rDNA regions involved in the formation of the stable preinitiation complex. To this end, a mouse S-100 extract was preincubated with 5′ or 3′ deletion mutants of mouse rDNA; a competitor gene was then added, and transcription was initiated by the addition of rXTPs. The 5′ borders of the regions involved in stable complex formation are identified in Fig. 5, in which the 5′ deletion mutants were used either in closed circular form (Fig. 5A) or after linearization to produce a 155-nucleotide runoff (Fig. 5B). The same qualitative results were obtained from these two kinds of assays, although the various borders have reproducibly somewhat different quantitative effects. 5′ deletions extending in to residue −149 (relative to the initiation site at +1 [Fig. 5A and B, lanes 1 and 2) inhibit transcription of the competitor template as efficiently as does the parental rDNA. 5′Δ − 126 allowed a small but reproducible increase in the amount of transcription of the competitor template (Fig. 5A, lanes 3 and 11; Fig. 5B, lane 3), and the 5′ deletions extending in to residue −40 allowed still more competitor transcription (Fig. 5A and B, lanes 4 through 6), indicating a reduced ability of these deletions to form a stable complex. Mutant 5′Δ − 35 is even less competent for stable complex formation, for it allowed yet more competitor transcription (an effect which is routinely approximately fivefold with linear templates [Fig. 5B, lane 7] but is of lesser magnitude with closed circular templates [Fig. 5A, lane 7]). Finally, 5′ deletions extending to or beyond residue −27 (Fig. 5A and B, lanes 8 and 9) exhibited no detectable stable complex formation, for they allowed control levels of competitor transcription. Thus, the primary 5′ border of the region involved in binding the essential transcription factors is at residue ~−35, while additional regions that extend upstream to residues ~−40, ~−100, and ~−140 further augment the stability of the complex.

The results of experiments that define the 3′ border of the rDNA region involved in stable complex formation are shown in Fig. 6. The 3′ deletion mutants were used in closed circular form (Fig. 6A), or they were linearized to produce an ~1,050-nucleotide runoff transcript (Fig. 6B). The 3′ deletions extending in to residue +9 efficiently inhibited synthesis from the competitor template (Fig. 6A, lanes 1 through 4; Fig. 6B, lanes 1 through 5), and thus they appear to contain the sequences needed to form a stable complex. However, a slight advantage in stable complex formation of closed circular 3′Δ+20 over 3′Δ+9 was consistently observed (Fig. 6A, lanes 3 and 4). Deletion 3′Δ+2 exhibits a markedly reduced capacity for complex formation, for it allowed a limited amount of synthesis from the competitor template (Fig. 6A, lane 5; Fig. 6B, lane 6), and 3′Δ − 5 and 3′Δ − 10 also had a reduced capacity for stable complex formation (Fig. 6A, lanes 6 and 7; Fig. 6B, lane 7). Finally, 3′Δ − 20 and more extensive 3′ deletions appeared quite incapable of stable complex formation (Fig. 6A, lanes 8 through 11; Fig. 6B, lane 8). Thus, the 3′ border of the minimal region involved in binding essential transcription factors is between positions −10 and −20, while sequences

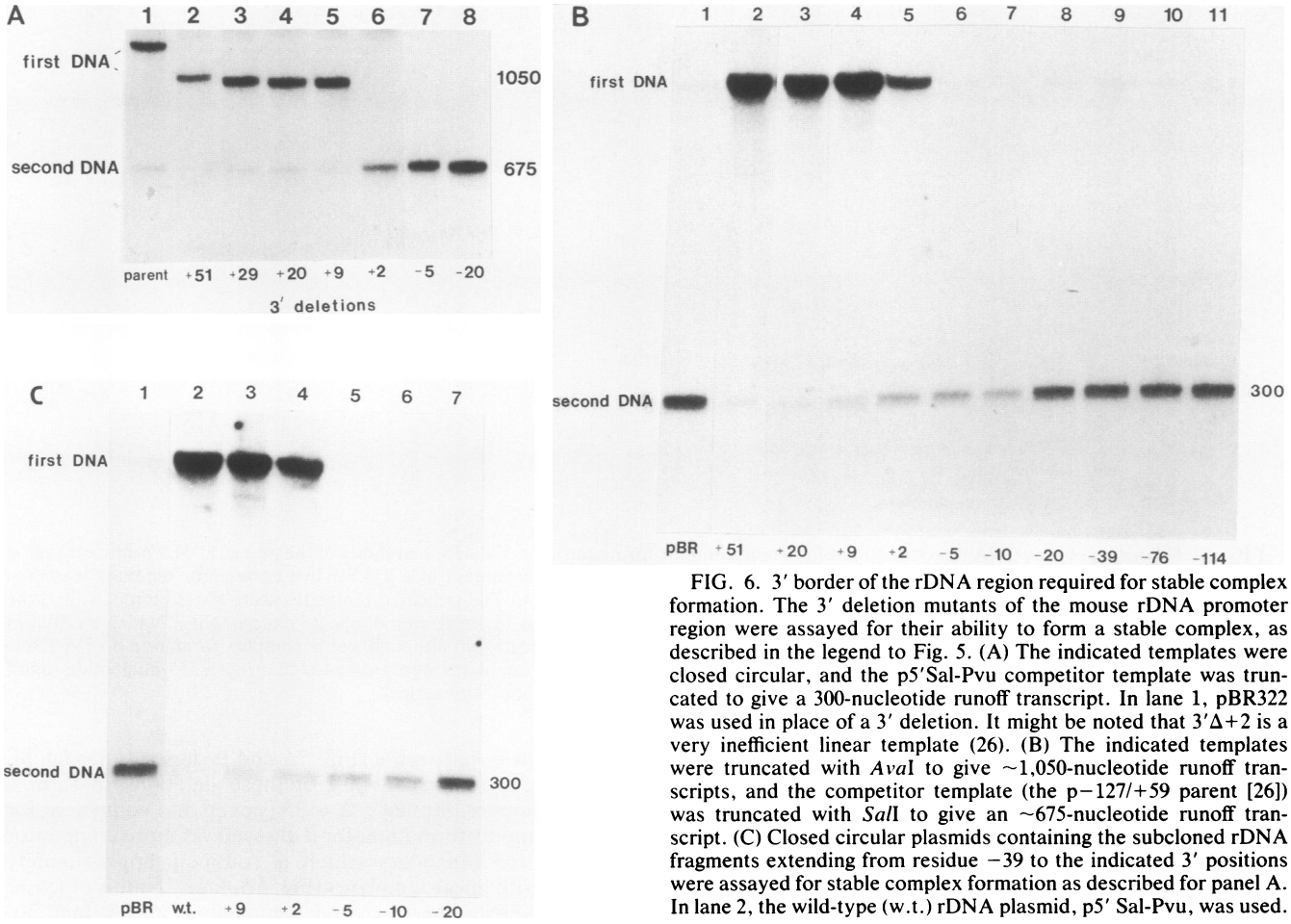

FIG. 6. 3′ border of the rDNA region required for stable complex formation. The 3′ deletion mutants of the mouse rDNA promoter region were assayed for their ability to form a stable complex, as described in the legend to Fig. 5. (A) The indicated templates were closed circular, and the p5′Sal-Pvu competitor template was truncated to give a 300-nucleotide runoff transcript. In lane 1, pBR322 was used in place of a 3′ deletion. It might be noted that 3′Δ+2 is a very inefficient linear template (26). (B) The indicated templates were truncated with *AvaI* to give ~1,050-nucleotide runoff transcripts, and the competitor template (the p−127/+59 parent [26]) was truncated with *SalI* to give an ~675-nucleotide runoff transcript. (C) Closed circular plasmids containing the subcloned rDNA fragments extending from residue −39 to the indicated 3′ positions were assayed for stable complex formation as described for panel A. In lane 2, the wild-type (w.t.) rDNA plasmid, p5′ Sal-Pvu, was used.

that extend downstream to position ~+2 augment the binding.

To directly ascertain the minimal region required for complex formation, various 3′ promoter deletions were subcloned to have additionally a 5′ deletion at residue −39. When assayed for their ability to form a stable complex (Fig. 6C), these mutants exhibited 3′ borders that are very reminiscent of the parental 3′ deletions; the major 3′ border was found to lie between residues −10 and −20 (lanes 6 and 7), although sequences extending to position ~+2 augmented complex stability (lanes 3 through 6). Thus, the subcloned rDNA region extending from residue −10 to −39 is sufficient for template commitment.

rDNA regions involved in binding factors D and D plus C. From the above results showing (i) that the minimal rDNA region needed to bind essential factors of the S-100 extract extends from position ~−35 to ~−15 (Fig. 5 and 6) and (ii) that factor D can stably bind the rDNA template in the absence of C (Fig. 4), one would infer that factor D is able to bind to the sequences residing between positions ~−35 and ~−15. This is directly addressed in the experiments shown in Fig. 7 by preincubation of the above-described deletions with fraction D before the addition of competitor template and fraction C. Figure 7A, in which the 3′ deletion mutants were preincubated with factor D, shows that the 3′ border of the D binding region is between residues −10 and −20; no effect of sequences downstream of this region was detected. This same result was obtained with the 3′ deletions that are also a 5′ deletion at residue −39 (Fig. 7B), for factor D bound efficiently to the subcloned rDNA region −39 to −10 but not to the subcloned region −39 to −20. When the 5′ deletions were preincubated with D (Fig. 7C), transcription of the competitor template was inhibited by the deletions extending in to residue −35 but not by 5′Δ − 27. These 5′ deletion analyses also suggest that D may bind with a slight preference to genes containing sequences upstream of residue −76 relative to genes lacking this region (Fig. 7A, lanes 3 and 4). Thus, factor D does indeed bind to the mouse rDNA promoter segment between positions −35 and −10, the minimal region that causes template commitment in the whole S-100 extract.

Finally, in light of the results indicating that factor D can bind efficiently to a 3′Δ − 10 (Fig. 7A and B) but that transcriptional initiation requires sequences that extend in the 3′ direction to between residues +2 and +9 (26), we examined whether these additional 3′ sequences (residues ~−10 to ~+9) were required for C binding. To this end, the 3′ deletion mutants were used for C binding analyses. In one reaction, both C and D were preincubated with the experimental template, while in a separate reaction, D was preincubated with a control runoff template. The reaction preparations were next mixed, and transcription was then initiated by addition of rXTPs. The relative level of transcription from the runoff templates (Fig. 8) is a measure of the relative stability of C binding to the 3′ deletion mutant-D complex. These results indicate that C binds to the complex of D and

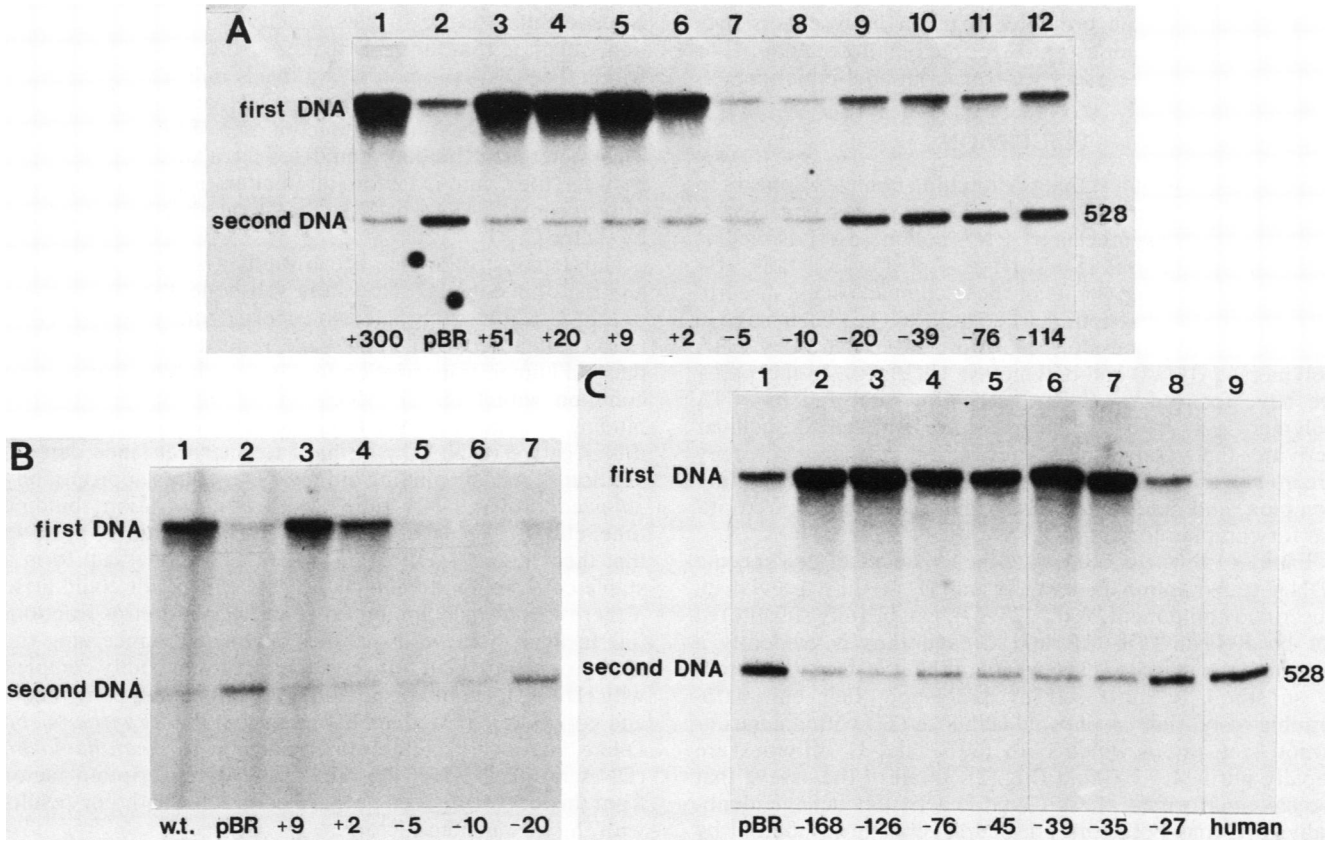

FIG. 7. 5′ and 3′ borders of the rDNA region required for binding transcription factor D. (A) 3′ border. The indicated closed circular 3′ deletions were preincubated with factor D at 30°C for 10 min. Reaction mixtures were then supplemented with a 528-nucleotide runoff competitor template and factor C, and a 3-min period of synthesis was initiated by the addition of rXTPs. In lane 1, the D was preincubated with pBR322. (B) Minimal binding region. Closed circular plasmids containing the subcloned rDNA fragment extending from residue −39 to the indicated 3′ positions were assayed for D binding described for panel A. w.t. indicates p5′Sal-Pvu. (C) 5′ border. The indicated closed circular 5′ deletions were assayed for D binding as described for panel A. In lane 9, the D was preincubated with a human rDNA template [pETS-RS(B)].

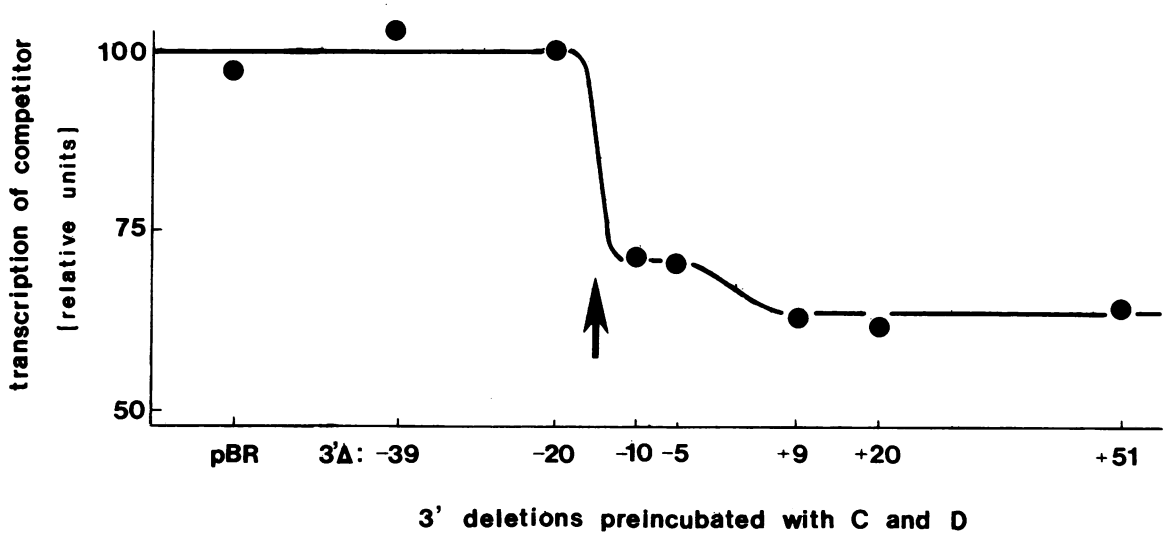

FIG. 8. 3′ border of the rDNA region required for C binding. The indicated closed circular 3′ deletion templates were preincubated with factors D and C at 30°C for 10 min. In separate reactions, a 528-nucleotide undeleted runoff template was preincubated with factor D. Reaction preparations were then mixed together and incubated for an additional 10 min, and finally a 3-min synthesis period was initiated by the addition of rXTPs. The amount of transcript from the 528-nucleotide runoff template was quantitated by densitometric scanning of the resultant autoradiographic bands and is shown in relative units.

5′Δ − 10. Thus, the promoter segment between residues ~−10 and ~+9 is not essential for the binding of either C or D, although it is necessary for transcriptional initiation.

DISCUSSION

The formation of a stable preinitiation complex appears to be a general feature of transcription in higher eucaryotes, characteristic of synthesis by RNA polymerase I as well as by RNA polymerases II and III (3, 7–9, 15, 22, 30, 34). By identifying the components of these complexes and discerning their modes of action, marked progress has been made in elucidating the process of transcriptional initiation by RNA polymerase III (18) and polymerase II (10, 30). In this paper we have focused on the transcription catalyzed by RNA polymerase I. In particular, we have isolated the cellular activities that catalyze rDNA transcription, shown how they are involved in forming the polymerase I stable transcription complex, and determined which rDNA sequences are critical for complex formation.

Figures 1 through 3 illustrate the existence of two specific rDNA transcription factors, C and D. A third previously described component, A (22, 23, 27), can be fully substituted for by RNasin (Fig. 1B and C) and thus is evidently a ribonuclease inhibitor. The purity of the transcription factors is substantially increased by preceding their chromatographic resolution on phosphocellulose (27) with a centrifugation step (8) in which both the C and D activities are rapidly purified ~100-fold (Fig. 2). In all of the assays that we have performed, these C and D activities behave identically to C and D factors purified several throusandfold by alternative chromatographic fractionations (Fig. 4).

At this point, mention should be made of the suggestion (4, 6) that fraction C may contain both polymerase I and a separate essential transcription factor, for such an eventuality would have significant bearing on the interpretation of the data presented here. We find that polymerase I activity (as assayed by nonspecific synthesis) coelutes with C activity upon gradient elution from phosphocellulose (Fig. 3A) and DEAE-Sephadex followed by heparin-sepharose (32) (the C fraction used in Fig. 4A), and this suggests that C activity may be RNA polymerase I. However, C can not simply be bulk polymerase I, for ~90% of the polymerase I activity of the extract is found in the supernatant fraction after the stable transcription complex is pelleted (Fig. 3A) (8), yet the supernatant is virtually devoid of C activity (Fig. 2B). Thus, either (i) fraction C contains a necessary activity in addition to the polymerase or (ii) C activity is a form of polymerase I. The evidence so far supports the latter possibility; we have further attempted to resolve polymerase I from a hypothetical second transcription factor in the C fraction by using a number of additional chromatographic resins, sucrose gradient centrifugation, and selective heat denaturation, yet in all of these methods, C behaves like polymerase I activity (32; J. Tower and B. Sollner-Webb, manuscript in preparation). Our most pure C activity is >80% pure in the polymerase I polypeptides. We thus believe that C activity is a subpopulation of polymerase I molecules that is activated to productively interact with the factor D-rDNA complex, and, therefore, C is enriched in the pellet fraction. This conclusion is also in agreement with results recently obtained by Paule et al. (29) using the protist *Acanthamoeba*.

Both mouse and human factor D can by themselves form a stable complex with the rDNA initiation region of the homologous species (Fig. 4) (22). This property, in conjunction with the observation of Mishima et al. (27) that factor D is responsible for the species selectivity of rDNA transcription, suggests that factor D makes species-specific contacts within the rDNA promoter, and this is indeed the case (Fig. 7). The binding of factor D to the rDNA template is in turn necessary for subsequent stable binding of factor C (Fig. 4). This synergistic binding could reflect a direct recognition between the C and D factors or an alteration of the topology of the promoter DNA by D so that it can then be recognized by factor C.

Our demonstration that D can stably associate with rDNA and that this complex allows the subsequent stable association of C can be contrasted to reports from other laboratories. (i) Learned et al. (19) have reported that a partially purified human transcription factor, which has properties in common with factor D, does not show sequence-specific binding to human rDNA template. However, we feel that this negative result may be hard to interpret since demonstration of specific binding of factor D requires appropriately adjusted protein/DNA ratios, KCl concentration, binding time, etc. (ii) The data of Meisfeld and Arnheim (22) indicate that their human fraction C (as well as their D) can form a stable complex on human rDNA, but this result could arise if there was a small amount of D activity in their C fraction. (iii) In light of these disparate conclusions concerning the ability of mammalian transcription factors to stably complex with rDNA, it should be noted that results obtained by Paule and co-workers (15), demonstrating that the *Acanthamoeba* D-like factor TIF-I stably associates with *Acanthamoeba* rDNA by itself while the other rDNA transcription factor from this organism does not, are consistent with our results with the mammalian factors.

To determine which mouse rDNA regions are involved in template commitment, we have assessed the ability of 5′ and 3′ deletion mutants to form a stable complex with the transcription factors of the mouse cell S-100 extract. A small core domain, extending from residues ~−35 to ~−15, was found to be essential for complex formation (Fig. 5 and 6). However, there are several additional domains that serve to sequentially increase the stability of the binding. On the 5′ side, the upstream borders of these domains are marked by the following pairs of deletions: −35 and −41, −76 and −126, and −126 and −147. On the 3′ side, complex stability is augmented by sequences that extend to between residues +2 and +9 (Fig. 5 and 6). Strikingly, each of these 5′ and 3′ domains that serve to enhance the stability of the transcription complex was previously identified as a domain of the mouse rDNA promoter that augmented the level of transcription under appropriately stringent reaction conditions (26). Presumably, these domains always augment binding, but only under the stringent conditions does complex stability limit transcriptional efficiency, and thus only then is the promoter assay sensitive to the role of these sequences.

Consistent with the results obtained with S-100 extract, stable complex assays with the substantially purified transcription factors identified this same core region as being required for the binding of factor D (Fig. 7). Factor C can also stably bind to this core region (~−35 to ~−15)-factor D complex (Fig. 8). The data further suggest that D binding may also be augmented by the upstream promoter domains (Fig. 7C), but they do not directly address how the rDNA segment from −10 to +9 may act.

This organization of the mouse rDNA sequences is quite similar to that reported for *Acanthamoeba* rDNA (1, 15, 16) in a number of respects. (i) By using the template commitment assay in whole S-100 extract, Paule and co-workers (15) have found that the region required for stable complex

formation is upstream of residue ~−20, while the sequences essential for transcription extend downstream to approximately the initiation site. (ii) Footprint analysis (1) has shown that this *Acanthamoeba* upstream domain binds to a D-like transcription factor, TIF-I, while the downstream sequences are only seen to interact in the presence of both the C-like active polymerase and TIF-I. (iii) Although Paule and co-workers have reported that the *Acanthamoeba* sequences required for stable complex formation (residues ~−48 to ~−19 [15]) extend significantly upstream of the region defined as the promoter (residues ~−32 to ~+9 [16]), we suggest that the complete *Acanthamoeba* rDNA promoter actually includes all of these segments but that their detection requires more stringent transcription conditions than were used in the promoter mapping studies (16). This interpretation would be consistent with our data showing that all of the mouse promoter domains serve to augment the stability of the transcription complex.

In conclusion, the data and comparisons presented in this paper strongly support the thesis that the process of rDNA transcription is highly conserved across eucaryotes. This is in contrast to the earlier interpretation that rDNA transcription was very different—possibly even fundamentally distinct—in various species, an interpretation based on (i) the sequences of the rDNA initiation region not being conserved across evolution (33) and (ii) in vitro transcription exhibiting species specificity (14). Initially, this concept appeared to be supported by reports that different rDNA promoters were of very different sizes, but recent studies indicate that these results are largely attributable to variations in assay conditions rather than basic differences in rDNA promoter size (26, 32). Furthermore, the internal organization of the rDNA core promoter is now seen to be highly conserved (32) between species as divergent as mammals (Fig. 5 through 7) and unicellular organisms (1, 15, 16). Finally, while initial results suggested that there were different numbers of essential rDNA transcription factors in different species (1, 4), the current study indicates that the number and mode of action of these factors is also preserved between mammals and unicellular organisms, again underscoring the basic similarity of the rDNA transcriptional process between different species.

ACKNOWLEDGMENTS

We thank Norman Arnheim for generously providing the human rDNA plasmids, Kathryn Miller for many helpful discussions, Jody Hejhall and Maria Isern for excellent technical assistance, and Sue Millionie for typing the manuscript.

This research was funded by the American Cancer Society and a Basil O'Conner Grant from the March of Dimes Birth Defects Foundation.

LITERATURE CITED

1. **Bateman, E., C. T. Iida, P. Kownin, and M. R. Paule.** 1985. Footprinting of ribosomal RNA genes by transcription initiation factor and RNA polymerase I. Proc. Natl. Acad. Sci. USA **82**:8004–8008.
2. **Birkenmeier, E. H., D. D. Brown, and E. Jordan.** 1978. A nuclear extract of *Xenopus laevis* oocytes that accurately transcribes 5S RNA genes. Cell **15**:1077–1086.
3. **Bogenhagen, D. F., W. M. Wormington, and D. D. Brown.** 1982. Stable transcription complexes of *Xenopus* 5S RNA genes: a means to maintain the differentiated state. Cell **28**:413–421.
4. **Buttgereit, D., G. Plugfelder, and I. Grummt.** 1985. Growth-dependant regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA). Nucleic Acids Res. **13**:8165–8180.
5. **Cavenaugh, A. H., P. K. Gokal, R. P. Lawther, and E. A. Thompson, Jr.** 1984. Glucocorticoid inhibition of initiation of transcription of the DNA encoding rRNA (rDNA) in lymphosarcoma P1798 cells. Proc. Natl. Acad. Sci. USA **81**:718–721.
6. **Cavanaugh, A. H., and E. A. Thompson, Jr.** 1985. Hormonal regulation of transcription of rDNA: glucocorticoid effects upon initiation and elongation in vitro. Nucleic Acids Res. **13**:3357–3369.
7. **Cizewski, V. C., and B. Sollner-Webb.** 1983. A stable transcription complex directs mouse ribosomal RNA synthesis by RNA polymerase I. Nucleic Acids Res. **11**:7043–7056.
8. **Culotta, V. C., R. Wides, and B. Sollner-Webb.** 1985. Eucaryotic transcription complexes are specifically associated in large sedimentable structures: rapid isolation of polymerase I, II, and III transcription factors. Mol. Cell. Biol. **5**:1282–1290.
9. **Davison, B. L., J.-M. Egly, E. R. Mulvihill, and P. Chambon.** 1983. Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature (London) **301**:680–686.
10. **Fire, A., M. Samuels, and P. A. Sharp.** 1984. Interactions between RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. **259**:2509–2516.
11. **Grummt, I.** 1981. Specific transcription of mouse ribosomal DNA in a cell-free system that mimics control in vivo. Proc. Natl. Acad. Sci. USA **78**:727–731.
12. **Grummt, I.** 1981. Mapping of a mouse ribosomal DNA promoter by in vitro transcription. Nucleic Acids Res. **9**:6093–6102.
13. **Grummt, I.** 1982. Nucleotide sequence requirements for specific initiation of transcription by RNA polymerase I. Proc. Natl. Acad. Sci. USA **79**:6908–6911.
14. **Grummt, I., E. Roth, and M. R. Paule.** 1982. Ribosomal RNA transcription is species specific. Nature (London) **296**:173–174.
15. **Iida, C. T., P. Kownin, and M. R. Paule.** 1985. Ribosomal RNA transcription: proteins and DNA sequences involved in preinitiation complex formation. Proc. Natl. Acad. Sci. USA **82**:1668–1672.
16. **Kownin, P., C. T. Iida, S. Brown-Shimer, and M. R. Paule.** 1985. The ribosomal RNA promoter of *Acanthamoeba castellanii* determined by transcription in a cell-free system. Nucleic Acids Res. **13**:6237–6248.
17. **Kurl, R. N., and S. T. Jacob.** 1985. Characterization of a factor that can prevent random transcription of cloned rDNA and its probable relationship to poly (ADP-ribose) polymerase. Nucleic Acids Res. **13**:89–101.
18. **Lasser, A. B., P. L. Martin, and R. G. Roeder.** 1984. Transcription of class III genes: formation of preinitiation complexes. Science **222**:740–748.
19. **Learned, R. M., S. Cordes, and R. Tjian.** 1985. Purification and characterization of a transcription factor that confers promoter specificity to human RNA polymerase I. Mol. Cell. Biol. **5**:1358–1369.
20. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA **77**:3855–3859.
21. **Miesfeld, R., and N. Arnheim.** 1982. Identification of the in vivo and in vitro origin of transcription in human rDNA. Nucleic Acids Res. **10**:3933–3949.
22. **Miesfeld, R., and N. Arnheim.** 1984. Species-specific rDNA transcription is due to promoter-specific binding factors. Mol. Cell. Biol. **4**:221–227.
23. **Miesfeld, R., B. Sollner-Webb, C. Croce, and N. Arnheim.** 1984. The absence of a human-specific ribosomal DNA transcription factor leads to nucleolar dominance in mouse>human hybrid cells. Mol. Cell. Biol. **4**:1306–1312.
24. **Miller, K. G., and B. Sollner-Webb.** 1981. Transcription of mouse rRNA genes by RNA polymerase I: in vitro and in vivo initiation and processing sites. Cell **27**:165–174.
25. **Miller, K. G., and B. Sollner-Webb.** 1982. Transcription of mouse ribosomal RNA genes, p. 69–100. *In* H. Busch (ed.), The Cell Nucleus, vol. 12. Academic Press, Inc., New York.

26. **Miller, K. G., J. Tower, and B. Sollner-Webb.** 1985. A complex control region of the mouse rRNA gene directs accurate initiation by RNA polymerase I. Mol. Cell. Biol. **5**:554–562.
27. **Mishima, Y., I. Financsek, R. Kominami, and M. Muramatsu.** 1982. Fractionation and reconstitution of factors required for accurate transcription of mammalian ribosomal RNA genes: identification of a species-dependent initiation factor. Nucleic Acids Res. **10**:6659–6669.
28. **Mishima, Y., O. Yamamoto, R. Kominami, and M. Muramatsu.** 1981. In vitro transcription of a cloned mouse ribosomal RNA gene. Nucleic Acids Res. **9**:6773–6785.
29. **Paule, M. R., C. T. Iida, P. J. Perna, G. H. Harris, D. A. Knoll, and J. M. D'Alessio.** 1984. In vitro evidence that eukaryotic ribosomal RNA transcription is regulated by modification of RNA polymerase I. Nucleic Acids Res. **12**:8161–8180.
30. **Safer, B., L. Yang, H. Tolunay, and W. F. Anderson.** 1985. Isolation of stable preinitiation, initiation, and elongation complexes from RNA polymerase II - directed transcription. Proc. Natl. Acad. Sci. USA **82**:2632–2638.
31. **Sollner-Webb, B., and S. L. McKnight.** 1982. Accurate transcription of cloned *Xenopus* rRNA genes by RNA polymerase I: demonstration by S1 nuclease mapping. Nucleic Acids Res. **10**:3391–3405.
32. **Sollner-Webb, B., and J. Tower.** 1986. Transcription of cloned ribosomal RNA genes. Annu. Rev. Biochem. **55**:801–830.
33. **Sommerville, J.** 1984. RNA polymerase I promoters and transcription factors. Nature (London) **310**:189–190.
34. **Wandelt, C., and I. Grummt.** 1983. Formation of stable preinitiation complexes is a prerequisite for ribosomal DNA transcription in vitro. Nucleic Acids Res. **11**:3795–3809.
35. **Weil, P., D. Luse, J. Segall, and R. Roeder.** 1979. Selective and accurate initiation of transcription of the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell **18**:464–484.
36. **Weil, P. A., J. Segall, B. Harris, S.-Y. Ng, and R. G. Roeder.** 1979. Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. J. Biol. Chem. **254**:6163–6173.
37. **Wilkinson, J. A., K. G. Miller, and B. Sollner-Webb.** 1983. Dinucleotide primers facilitate convenient identification of the mouse ribosomal DNA transcription site. J. Biol. Chem. **258**:13919–13928.
38. **Yamamoto, O., N. Takakusa, Y. Mishima, R. Kominami, and M. Muramatsu.** 1984. Determination of the promoter region of mouse ribosomal RNA gene by an in vitro transcription system. Proc. Natl. Acad. Sci. USA **81**:244–303.