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# Eucaryotic Transcription Complexes Are Specifically Associated in Large Sedimentable Structures: Rapid Isolation of Polymerase I, II, and III Transcription Factors

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**RNA synthesis in eucaryotes takes place on template molecules that are activated by stably associating with limiting transcription factors. In this paper we demonstrate that such stable transcription complexes can be specifically sedimented from in vitro transcription reaction mixtures by mild centrifugation. This occurs with stable complexes of genes transcribed by all three classes of eucaryotic RNA polymerase and with S-100 as well as whole-cell extracts. However, the transcriptional capacity of the isolated complex differs for the three polymerase classes. The pelleted ribosomal DNA (polymerase I) complex contains all the factors necessary for transcription, each purified 25- to 50-fold, whereas the pelleted adenovirus major late promoter (polymerase II) complex lacks a factor that remains in the supernatant. In the case of 5S DNA (polymerase III), a necessary factor associates slowly with the sedimentable complex. Notably, the interactions responsible for this rapid sedimentation are specific for DNA molecules in stable complexes, suggesting that the in vitro sedimentable complex mirrors the in vivo structural organization of active genes.**

Eucaryotic transcription factors stably associate with the DNA molecules that serve as templates for RNA synthesis. Formation of such stable transcription complexes has been demonstrated in vitro with genes transcribed by all three classes of polymerase (1, 3, 5–7, 9, 11, 22, 24, 25, 27) and thus appears to be the general means of maintaining the activated state of eucaryotic genes. The constituents of these stable complexes and their functional roles are currently under considerable investigation. The DNA sequences involved in stably binding polymerase I, II, and III transcription factors have been delineated for genes that encode 45S rRNA (B. Sollner-Webb, J. Tower, V. Culotta, and J. Windle, *in* J. Setlow and A. Hollaender, ed., *Genetic Engineering*, in press), the adenovirus major late transcript (7), and 5S RNA (21), respectively. In all cases, stable complex formation utilizes the same template region that was previously identified as the promoter. Considerable progress has also been made in isolating and characterizing the proteins involved in the transcription complex. In many cases, however, these studies have been hindered by the difficulty in purifying these factors without effecting significant losses of activity; despite much effort, most eucaryotic transcription factors have only been purified to a limited extent.

As an alternative to traditional chromatographic methods, we have utilized a biological fractionation as the first step in purifying transcription factors, taking advantage of their specific binding to template molecules. We show that stable complexes and the associated transcription factors can be quantitatively and selectively sedimented from in vitro transcription reaction mixtures, whereas the bulk of the extract protein remains soluble. This procedure is effective in isolating transcription complexes of all three classes of eucaryotic polymerase.

## MATERIALS AND METHODS

**Transcription by polymerase I. (i) Mouse system.** S-100 extracts were prepared, as previously described (15), from log-phase mouse L1210 tissue culture cells. Transcription reaction mixtures of 25 μl contained 5 to 7 μl of S-100 extract and were made up of 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 90 mM KCl, 5 mM $MgCl_2$, and 300 μg of α-amanitin per ml. Where indicated, the S-100 was replaced by 3 to 5 μl of a whole-cell extract prepared, as described by Manley et al. (12), from the L1210 cells. Reaction mixtures were also supplemented with mouse ribosomal DNA (rDNA) template (7 to 10 μg/ml) (p5′*Sal-Pvu*, cloned rDNA nucleotides −170 to +300; 15) truncated with either *Sma*I at nucleotide +155 or *Pvu*II at position +300 to generate the runoff templates p155 and p300. Before centrifugal fractionation, the extract was preincubated with the template for 45 min at 30°C in the absence of added ribonucleotide triphosphates (rXTPs) (6). Reaction mixtures were then centrifuged at 15,000 × *g* for 5 min at 4°C, and supernatants were removed carefully to avoid disturbing the invisible pellets. Pellets were washed by gentle rinsing with 25 μl of chilled transcription buffer (15 mM HEPES [pH 7.9], 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 90 mM KCl, 5 mM $MgCl_2$) and then were respun or, alternatively, were sedimented through 25% glycerol cushions. Pellets were either reconstituted with supernatant or suspended in 25 μl of transcription buffer which also contained 300 μg of α-amanitin per ml. All reaction mixtures were supplemented with competitor template (final concentration, 10 μg/ml) to permit detection of any exchange or reassembly of transcription complexes, and rRNA synthesis was initiated by the addition of 500 μM each ATP, GTP, and UTP and 50 μM [α-$^{32}$P]CTP (2.5 Ci/mmol). Incubations were carried out at 30°C for 10 to 15 min. In mouse S-100 extract reaction mixtures preincubated for 45 min, transcription began immediately on addition of rXTPs to the stable complex, and the bulk of the rRNA synthesis occurred in the first 10 min of nucleotide addition (data not shown). Nucleic acids were purified, and the runoff transcripts were analyzed by polyacrylamide gel electrophoresis and autoradiography as previously described (15).

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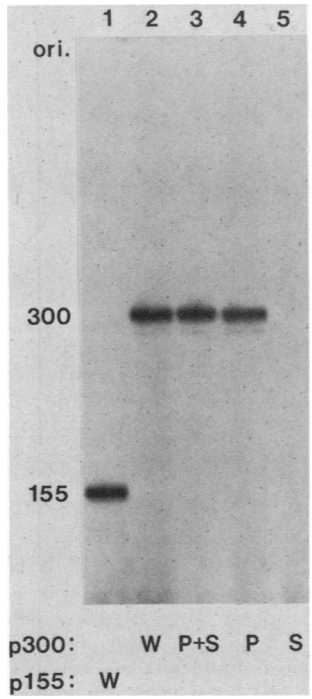


FIG. 1. Transcriptional capacity of supernatant and pellet fractions derived from mouse rDNA preincubated with mouse S-100 extract. Reaction mixtures containing 5 μl of mouse S-100 extract and 8 μg of p300 template per ml were preincubated for 45 min in the absence of rXTPs and centrifuged. The designated fractions were supplemented with p155 competitor, [α-$^{32}$P]CTP, and rXTPs and incubated for 15 min at 30°C. The autoradiogram shows the resolved transcripts from: (lane 1) W, uncentrifuged whole reaction mixture; (lane 2) P+S, pellet suspended in supernatant; (lane 3) P, pellet suspended in buffer; (lane 4) S, supernatant. ori., Origin of gel.

**(ii) Human system.** Whole-cell extracts, kindly provided by Jeff Corden or prepared in our laboratory, were made from HeLa cells by the procedure of Manley et al. (12). Before assay, insoluble components of the extract were removed by centrifugation of undiluted extract at 4,000 × *g* for 5 min. Transcription reactions (25 μl) contained 5 to 7 μl of extract, 7.5 to 10 μg of human rDNA per ml, and the same final contents as the mouse S-100 extract reaction mixtures. Where indicated, 5 to 7 μl of a human S-100 extract derived from HeLa cells replaced the whole-cell extract. The human rDNA template was pETS-RB(S) (cloned rDNA nucleotides −425 to +700, kindly provided by Norman Arnheim; 13) truncated at position +700 with *Sal*I. Human transcription complexes were assembled and fractionated as described above.

**Transcription by polymerase II.** RNA polymerase II transcription reaction mixtures of 25 μl contained 7.5 μl of the prespun HeLa whole-cell extract (see human polymerase I system) and 50 μg of template DNA per ml in a buffer containing 10 mM Tris (pH 7.9), 8% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 6.25 mM $MgCl_2$, and 60 mM KCl (12). The template DNA, a kind gift from Jeff Corden, was the cloned initiation region of the adenovirus 2 major late transcription unit (nucleotides −250 to +554) truncated either at position +554 with *Bam*HI or at position +405 with *Hinc*II. Extract and template were preincubated for 10 to 60 min at 30°C, and the reaction mixtures were then centrifuged as described above for polymerase I. Pellets were suspended in either polymerase II reaction buffer or supernatant. Reaction mixtures were then supplemented with labeled rXTPs and incubated for 45 min at 30°C. Transcription in these polymerase II reaction mixtures began immediately on complex formation and proceeded for 30 to 45 min at a gradually declining rate (data not shown). Reaction mixtures were then processed as described above.

**Transcription by polymerase III.** S-100 transcription reaction mixtures were set up, fractionated, and assayed as described above for polymerase I reaction mixtures except that α-amanitin was omitted and ATP, GTP, and UTP were at 400 rather than 500 μM. The template (3) was a closed circular plasmid containing a *Xenopus borealis* 5S RNA gene (pXBS1) or a maxigene derivative (p+115/+77), both generously provided by Donald Brown. The preincubation period preceding centrifugal fractionation was varied between 10 and 90 min as indicated. Pellets were suspended in either polymerase III transcription buffer or supernatant. All reactions were then supplemented with labeled rXTPs and competitor DNA, incubated for 90 min at 30°C, and processed as described above.

**Polymerase I assay and other methods.** To assess total RNA polymerase I activity, fractions were incubated in 20-μl reaction mixtures containing 1.5 mM $MnCl_2$, 1 mM $MgCl_2$, 30 mM HEPES (pH 7.9), 500 μM each ATP, CTP, and GTP, 50 μM [α-$^{32}$P]UTP (2 Ci/mmol), 75 mM KCl, 300 μg of α-amanitin per ml, 0.2 mg of bovine serum albumin per ml, 0.5 mM dithiothreitol, and 100 μg of sonicated calf thymus DNA per ml (23). The small amount of background nontranscriptional incorporation was measured in parallel reaction mixtures also containing 100 μg of actinomycin D per ml. After incubation at 30°C for 30 min, samples were precipitated with trichloroacetic acid, and incorporation was quantitated by scintillation counting.

Protein concentration was measured by Coomassie staining (4) as directed by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Sodium dodecyl sulfate gel electrophoresis and silver staining followed the methods of Laemmli (10) and Morrissey (17), respectively.

The approximate sedimentation coefficient of the rDNA transcription complex was determined from its rate of pelleting relative to that of purified 45S rRNA sedimented under similar conditions.

## RESULTS

**Isolation of polymerase I preinitiation complexes.** To study the stable transcription complexes that form on mammalian rRNA genes (6, 25), we used a mouse S-100 extract and linearized mouse rDNA templates. This rDNA was truncated either with *Sma*I (p155), which directs the synthesis of a 155-nucleotide transcript, or with *Pvu*II (p300), which generates a 300-nucleotide transcript (6, 15). When reaction mixtures were preincubated with DNA molecules of one size before the addition of the other competitor template and the initiation of synthesis, RNA was exclusively transcribed from the template added first (Fig. 1, lanes 1 and 2); this is diagnostic of stable complex formation on the first template added to an in vitro reaction mixture (6).

We have developed a simple centrifugation procedure to purify the activated rDNA template with its stably associated transcription factors from in vitro synthesis reaction mixtures. Preinitiation complexes were first formed by incubation of rDNA and the S-100 extract in the absence of rXTPs for 30 to 45 min, and the reaction mixtures were then centrifuged at 15,000 × *g* for 5 min. The synthetic activity of the p300-derived supernatant and suspended pellet was assayed in the presence of p155 competitor after the addition

TABLE 1. Partitioning of transcriptional activity, total protein, and polymerase I activity during centrifugal fractionation of S-100 reaction mixtures with mouse rDNA[a]

| Parameter | % of parameter present in[b]: | |
|---|---|---|
| | Pellet | Supernatant |
| Transcriptional activity | >90 | <2 |
| Total protein content | 2–4 | 96–98 |
| RNA polymerase I activity | ~10 | ~90 |

[a] Complete reaction mixtures, pellets, and supernatants were prepared and assayed as described in the legend to Fig. 1. Protein content was determined both by Coomassie staining in solution (4) and by silver staining of sodium dodecyl sulfate protein gels (10, 17); the values for different extracts varied within the indicated ranges. Total polymerase I activity was determined as described in the text (23).

[b] Values are expressed relative to that of an unfractionated reaction as 100%.

of rXTPs and [α-$^{32}$P]CTP (Fig. 1). Centrifugation caused no loss of transcriptional activity or complex stability, since the pellet reconstituted with supernatant (lane 3) transcribed RNA at the level of the unfractionated reaction mixture (lane 2) and there was no synthesis from the competitor template. Strikingly, the pellet suspended in buffer catalyzed this same level of rRNA synthesis even in the absence of supernatant (lane 4), whereas the isolated supernatant was virtually devoid of activity (lane 5). Thus, the active rDNA template and all the necessary transcription factors cosediment on centrifugation of an in vitro reaction mixture.

This centrifugal isolation of the stable complex afforded substantial purification of polymerase I transcription factors. The protein concentration of pellet and supernatant fractions was determined by Coomassie staining in solution and by silver staining of sodium dodecyl sulfate protein gels. Both methods demonstrated that the pellet contained only 2 to 4% of the total extract protein, whereas the supernatant retained

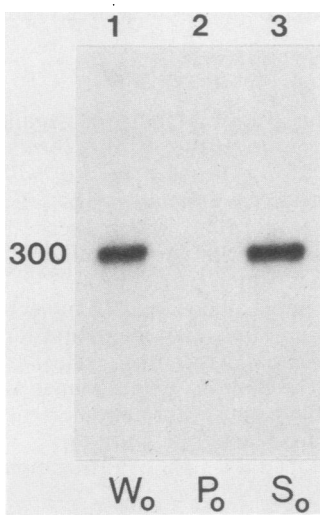


FIG. 2. Synthetic capacity of pellet and supernatant fractions formed in the absence of template DNA. Reaction mixtures were preincubated and centrifuged as described in the legend to Fig. 1, but template DNA was absent. The resultant pellet ($P_o$, lane 2) and supernatant ($S_o$, lane 3), as well as whole reaction mixtures ($W_o$, lane 1), were supplemented with p300 template and assayed for transcriptional capacity as described in the legend to Fig. 1.

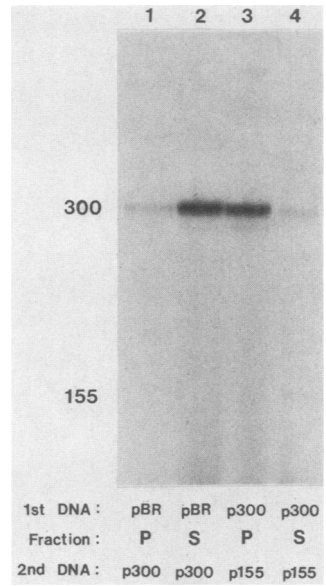


FIG. 3. rDNA sequences are required for sedimenting polymerase I transcriptional activity. Mouse S-100 extract was preincubated at 30°C for 30 min with 7.5 μg of either pBR322 (lanes 1 and 2) or p300 (lanes 3 and 4) per ml. Both DNAs were cleaved as indicated in the text. Reaction mixtures were then subjected to centrifugal fractionation. The suspended pellets and the supernatants were supplemented with 7.5 μg of either p300 (lanes 1 and 2) or p155 competitor (lanes 3 and 4) per ml and incubated for 30 min at 30°C before a 15-min labeling period was initiated by the addition of rXTPs. Lanes: 1, pBR322-derived pellet; 2, pBR322-derived supernatant; 3, p300-derived pellet; 4, p300-derived supernatant. P, Pellet; S, supernatant.

96 to 98% (Table 1). Centrifugation thus effects a rapid 25- to 50-fold purification of the limiting factors required for accurate rRNA synthesis.

To investigate whether RNA polymerase I was sequestered with the pelleted active template, we determined the distribution of this enzymatic activity from the level of α-amanitin-resistant and actinomycin D-sensitive transcription catalyzed with sheared calf thymus DNA (Table 1). The supernatant contained 90% of the polymerase I activity, whereas the pelleted preinitiation complex cosedimented with 10%. Thus, only a small percentage of polymerase I in the S-100 extract is required for maximal levels of specific transcription.

Sedimentation of RNA polymerase I transcription factors is a template-mediated event. Figure 2 shows an experiment in which the S-100 transcription reaction mixture was preincubated and centrifuged as described in the legend to Fig. 1, except that the rDNA template was omitted. The resulting supernatant and pellet were then supplemented with p300 DNA and rXTPs, and the transcriptional activity was assayed. In this case, the supernatant retained the full synthetic capacity of the parent reaction mixture, whereas the pellet was devoid of activity (Fig. 2). Thus, the essential transcription factors are inherently soluble under the reaction conditions and only sediment when associated with the template.

We investigated whether the sedimentation of transcription factors was actually dependent on stable complex formation or if nonspecific associations with nucleotide sequences other than the polymerase I promoter would similarly afford their rapid sedimentation. The pBR322 vec-

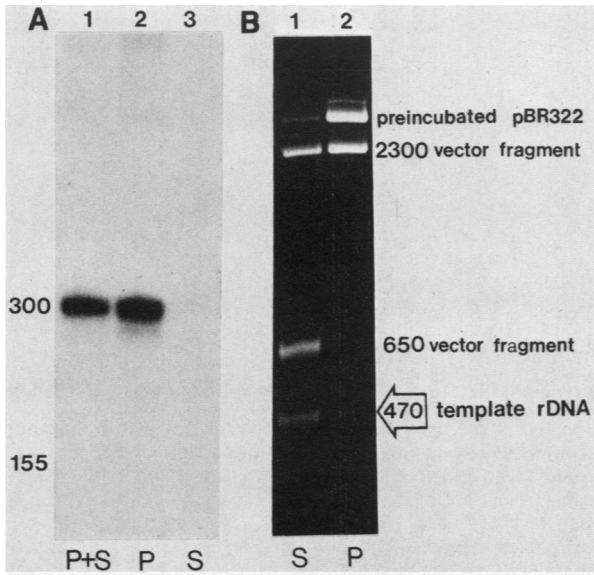


FIG. 4. Selective precipitation of active rRNA genes. Mouse S-100 extract was preincubated with 7.5 μg of pBR322 per ml for 10 min at 30°C in the absence of rXTPs. rDNA template (7.5 μg/ml), cleaved as indicated in the text, was then added and incubation was continued for 45 min before centrifugation. (A) The fractions were assayed for transcriptional capacity as described in the legend to Fig. 1. Lanes: 1, pellet suspended in supernatant; 2, pellet suspended in buffer; 3, supernatant. (B) DNA purified from 10 reaction mixtures worth of the indicated fractions was resolved by acrylamide gel electrophoresis and visualized by ethidium bromide staining. Lanes: 1, supernatant; 2, pellet. An arrow marks the 470-base-pair rDNA template. P, Pellet; S, supernatant; P+S, pellet suspended in supernatant.

tor and p300 rDNA were compared for their abilities to pellet the rRNA transcriptional activity (Fig. 3). Since polymerase I transcription factors appear to be DNA-binding proteins (14; J. Tower and B. Sollner-Webb, unpublished observations), we attempted to favor overall DNA solubility by using shorter DNA fragments. Both DNAs were digested with *Hin*fI and *Hin*dIII to generate a series of fragments of ≤1.1 kilobases; the resultant template fragment (containing rDNA residues −170 to +300) was 500 base pairs in length. Extract was preincubated with either cleaved pBR322 (lanes 1 and 2) or cleaved rDNA (lanes 3 and 4), and the reaction mixtures were subjected to centrifugal fractionation. The synthetic activity of the fractions derived from the pBR322-preincubated reaction mixture was measured by supplementing the suspended pellet and the supernatant with p300 rDNA since the transcription factors do not stably bind pBR322 DNA (6). The fractions derived from the rDNA-preincubated reaction mixture were transcribed in the presence of additional p155 competitor template to assess complex stability. As expected, the transcriptional activity of the rDNA-preincubated reaction mixture was recovered in the pellet as a stable complex (lanes 3 and 4). However, in the reaction mixture preincubated with pBR322 DNA, the transcriptional activity remained in the supernatant (lanes 1 and 2). Thus, the polymerase I transcription factors selectively sedimented when bound to an rDNA template in a stable transcription complex but were not sedimented after incubation in a similar reaction mixture with DNA lacking the rDNA promoter.

Since only a small fraction of the rDNA molecules in an S-100 extract transcription reaction mixture actually serves as the template for rRNA synthesis (6), we next asked whether these active template molecules were selectively pelleted by centrifugation. To favor detecting a selective sedimentation of template molecules, we introduced two modifications that increased the solubility of nontemplate rDNA. First, cell extract was preincubated with pBR322 before the addition of the template to help adsorb DNA-binding proteins that otherwise cause the input rDNA to sediment nonspecifically. As a second method of increasing the solubility of the bulk rDNA, a smaller template was used. (This 470-base-pair fragment [nucleotides −170 to +300], as well as 625- and 2,300-base-pair fragments of

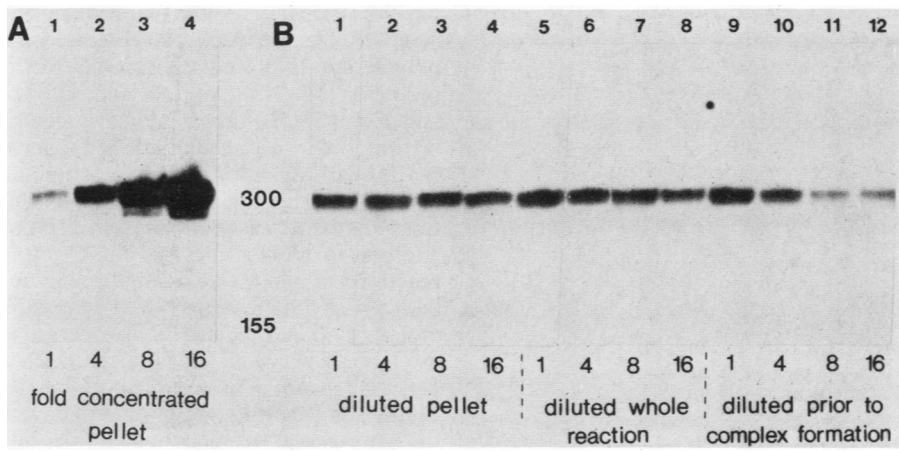


FIG. 5. Transcriptional capacity of concentrated and diluted polymerase I stable complexes. (A) Preinitiation complexes formed with the p300 template were pelleted from the indicated increasing number of normal (25-μl) reaction mixtures and suspended in 25 μl of buffer. Transcriptional capacity was then assayed as described in the legend to Fig. 1. (Lane 1) 1 pellet; (lane 2) 4 pellets; (lane 3) 8 pellets; (lane 4) 16 pellets. (B) Preinitiation complexes were formed with the p300 template in 25-μl transcription reaction mixtures as described in the legend to Fig. 1 and were either isolated by sedimentation (lanes 1 through 4) or retained as unfractionated reactions (lanes 5 through 8). The complexes were then diluted with transcription buffer as indicated. In lanes 9 through 12, unfractionated reaction mixtures were diluted with buffer before template was added and the formation of the preinitiation complex was allowed during a 45-min incubation period. The synthetic capacity of all samples was then measured after the addition of competitor template and rXTPs as described in the legend to Fig. 1. (Lanes 1, 5 and 9) undiluted reaction mixtures; (lanes 2, 6, and 10) diluted 4-fold; (lanes 3, 7, and 11) diluted 8-fold; (lanes 4, 8, and 12) diluted 16-fold.

vector DNA, was generated by *SalI* and *Eco*RI cleavage of the p300 DNA.) When a reaction mixture formed under these conditions was centrifuged, the transcriptional activity was still recovered completely in the pellet (Fig. 4A, lane 2). However, this pellet contained only 10 to 15% of the input 470-base-pair template fragment (Fig. 4B). Thus, all of the rDNA that assembled into a stable transcription complex was pelleted by centrifugation, whereas the bulk of the rDNA was transcriptionally inactive and remained soluble.

Full synthetic activity was maintained after substantial concentration or dilution of the pelleted active template. Preinitiation complexes formed with p300 rDNA were sedimented from large reaction mixtures and were suspended at concentrations of up to 16 times that of a normal transcription reaction mixture (Fig. 5A). The corresponding rRNA signal increased linearly with the amount of pellet. The stability of the isolated transcription complex to dilution is shown in Fig. 5B. Here, the sedimented preinitiation complexes (lanes 1 to 4), like those present in complete S-100 extract reaction mixtures (lanes 5 to 8), maintained high transcriptional activity when diluted up to 16-fold with buffer. In contrast, S-100 extract reaction mixtures diluted before the formation of the stable complex exhibited substantial losses of activity (lanes 9 to 12). The transcription complexes that did form on incubation with rDNA in these diluted reaction mixtures (lanes 9 to 12), like those formed on template before dilution (lanes 1 to 8), were stable, for they prevented synthesis from subsequently added p155 competitor template.

Finally, the selective sedimentation of transcription complexes is not unique to the S-100 system or to mouse templates. Stable mouse rDNA complexes were similarly formed and pelleted in reaction mixtures containing a whole-cell extract derived from mouse tissue culture cells (data not shown). Moreover, activated templates were quantitatively sedimented from reaction mixtures containing human rDNA and either S-100 (data not shown) or whole-cell extracts (Fig. 6) derived from HeLa cells. Thus, centrifugation appears to be a generally applicable method for isolating activated rDNA templates and their associated factors from various in vitro transcription reaction mixtures.

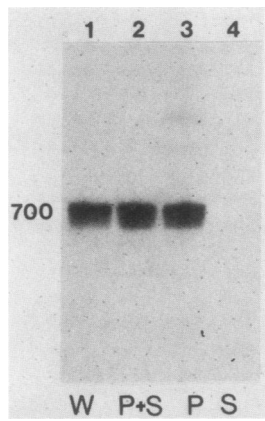


FIG. 6. Transcriptional capacity of centrifugal fractions derived from reaction mixtures of human rDNA in a human whole-cell extract. Reaction mixtures containing 5 μl of HeLa whole-cell extract and 7.5 μg of human rDNA per ml were incubated for 45 min in the absence of rXTPs and then centrifuged. The designated fractions were then supplemented with [α-$^{32}$P]rXTPs and incubated at 30°C for 30 min. Lanes: 1, uncentrifuged whole reaction mixtures; 2, pellet suspended in supernatant; 3, pellet suspended in buffer; 4, supernatant. W, whole reaction mixture; P, pellet; S, supernatant; P+S, pellet suspended in supernatant.

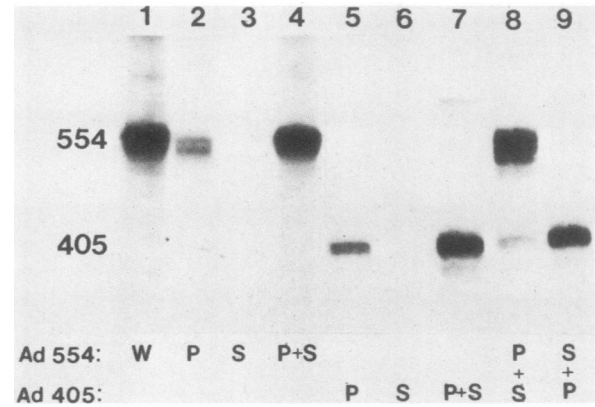


FIG. 7. Polymerase II: centrifugal fractionation of the preinitiation complex. The major late transcription unit of adenovirus 2, truncated at position +554 (lanes 1 through 4) or +405 (lanes 5 through 7), was preincubated with HeLa whole-cell extract in the absence of rXTPs for 10 min at 30°C. Reaction mixtures were centrifuged and the resultant fractions were assayed for transcriptional capacity by supplementation with [α-$^{32}$P]CTP, rXTPs, and additional adenovirus template. Lanes: 1, whole, uncentrifuged reaction mixtures; 2 and 5, pellet suspended in buffer; 3 and 6, supernatant; 4 and 7, pellet reconstituted in its own supernatant. To determine which centrifugal fraction contained the activated DNA, we suspended the Ad554 pellet in Ad405 supernatant (lane 8) and the Ad405 pellet in Ad554 supernatant (lane 9). The same results were obtained with reaction mixtures that were preincubated for 60 min. W, Whole reaction mixture; P, pellet; S, supernatant; P+S, pellet suspended in supernatant.

**Centrifugal isolation of polymerase II transcription complexes.** To investigate whether the rapid sedimentation procedure also could be applied to RNA polymerase II transcription systems, we used adenovirus II DNA and a whole-cell extract derived from HeLa cells (12). The template consisted of the subcloned initiation region of the adenovirus major late transcription unit truncated at either position +554 or +405 to produce 554-or 405-nucleotide runoff RNAs, respectively (Fig. 7, lanes 1 and 7). Stable polymerase II preinitiation complexes were assembled by preincubation of the extract and template in the absence of added rXTPs (7); this treatment indeed prevented the transcription of a subsequently added competitor template (data not shown). Reaction mixtures containing such preinitiation complexes were then centrifuged as described above, and fractions were assayed for transcriptional activity by the addition of rXTPs and [α-$^{32}$P]CTP. As with polymerase I reactions, a pellet reconstituted with supernatant was fully active (Fig. 7, lanes 4 and 7), and the supernatant was devoid of activity (lanes 3 and 6). However, in the case of polymerase II, only very low levels of synthesis were directed by the pellet suspended in transcription buffer (lanes 2 and 5). These same results were obtained with all preincubation times examined (from 10 to 60 min), and pulse-chase experiments demonstrated that the apparent inactivity of this pellet fraction was not attributable to rapid degradation of the transcript (data not shown). Thus, the components essential for polymerase II transcription appear to divide between pellet and supernatant when reaction mixtures containing the preinitiation complex are centrifuged.

To determine which centrifugal fraction contained the bulk of the activated adenovirus template, we cross-

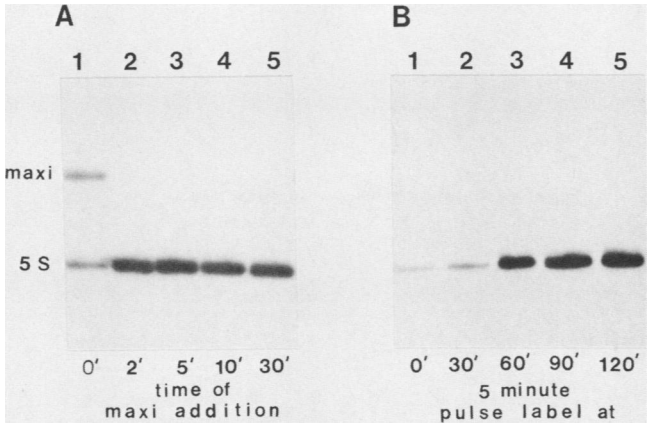


FIG. 8. Polymerase III: kinetics of stable complex formation and onset of 5S RNA synthesis. (A) Polymerase III transcription reaction mixtures containing 10 μg of 5S DNA template per ml and the mouse S-100 extract were incubated for the indicated lengths of time at 30°C before the addition of 10 μg of competitor 5S maxigene per ml, [α-$^{32}$P]CTP, and rXTPs to initiate a 60-min synthesis period. Transcriptional activity was assayed from reactions in which (lane 1) the 5S and maxigene templates were added simultaneously and (lanes 2 through 5) the 5S template was preincubated for 2, 5, 10, and 30 min before maxigene addition. (B) 5S DNA template was preincubated with S-100 extract in the absence of rXTPs for the indicated lengths of time. A 5-min synthesis period was initiated by addition of [α-$^{32}$P]CTP and rXTPs to reaction mixtures after preincubation periods of (lane 1) 0 min, (lane 2) 30 min, (lane 3) 60 min, (lane 4) 90 min, and (lane 5) 120 min.

combined pellets formed using one runoff template with supernatants formed using the other runoff DNA. In both cross-combinations, full synthesis was restored, the specificity of which was determined by the pellet (Fig. 7, lanes 8 and 9). Thus, the active adenovirus template with its stably associated transcription factor(s) quantitatively sediments to the pellet. In contrast to results obtained with polymerase I, however, an additional polymerase II transcription factor(s) evidently remains soluble.

**Centrifugal isolation of polymerase III transcription complexes.** To investigate the third eucaryotic RNA polymerase class, we transcribed an *X. borealis* 5S RNA gene in the mouse S-100 extract, a system previously shown to initiate and terminate accurately (26). The stable 5S preinitiation complex (3, 5) formed rapidly, since preincubation of the extract with the 5S RNA gene for 2 min precluded transcription of a subsequently added competitor maxigene (Fig. 8A). This maxigene has a 39-base-pair insertion 3′ to the promoter and thus synthesizes a transcript 39 nucleotides longer than normal 5S RNA (3). Unlike the results obtained in experiments performed with polymerases I and II, in which transcription began immediately on formation of the stable complex, maximal rates of 5S RNA synthesis were attained only after 60 to 90 min of incubation. This is demonstrated by preincubating the 5S RNA for various lengths of time before rXTPs were added to initiate a 5-min labeling period (Fig. 8B). Such rapid formation of the stable complex and delayed onset of maximal transcription is in good agreement with results reported for synthesis of *Xenopus* 5S RNA in both frog (2, 3) and human (11) cell extracts.

In an attempt to isolate the stable 5S transcription complex, we preincubated reaction mixtures with the cloned 5S gene or the maxigene and subjected them to centrifugal fractionation. Both the transcriptionally inactive and active stable complexes were investigated by varying the preincubation time preceding centrifugation from 10 to 90 min. To determine which fraction contained the stable complex, we cross-combined the resultant pellets and supernatants—the 5S pellet with the maxigene supernatant (Fig. 9, lanes 1) and vice versa (lanes 2). In all cases, ≥90% of the synthesis was directed by the template of the pellet. Thus, the stable polymerase III preinitiation complex, like those in polymerase I and II reactions, sediments to the pellet after mild centrifugation.

Despite the efficient sedimentation of the 5S preinitiation complex at all of the times examined (Fig. 9, lanes 1 and 2), the ability of the pellet to transcribe in the absence of soluble factors varied with the time of preincubation. After 90 min, all the necessary factors cosedimented, and the pellet exhibited the synthetic activity of the unfractionated reaction mixture (Fig. 9A, lanes 4 and 5). However, with shorter preincubation periods, the pelleted template synthesized RNA with decreased efficiency (Fig. 9B and C, lanes 4), and after a 10-min preincubation period, the pellet was virtually devoid of activity (Fig. 9D, lane 4). These results indicate that a necessary polymerase III transcription factor associates slowly with the stable preinitiation complex, cosedimenting after a 90-min preincubation period but remaining soluble at shorter times.

Data in Fig. 9 and 10 indicate that the slowly associating polymerase III transcription factor limited the overall level of 5S RNA synthesis in our in vitro system. As noted above, only a small percentage of the activated templates were in the supernatant (Fig. 9, lanes 1 and 2). However, after short incubation periods, these templates directed considerably more synthesis when reacted alone (Fig. 9C and D, lanes 3) than when combined with the additional stable complexes of the pellet (Fig. 9C and D, lanes 1 and 2). This suggests that the active templates competed for a limiting factor from the supernatant. Moreover, the slowly associating transcription factor was indeed absent from the supernatant prepared after a 90-min incubation period, for this fraction did not stimulate synthesis from a pellet prepared after a 10-min preincubation period (Fig. 10). In fact, there was a close correlation between the time required for this slow-associating factor to cosediment with active template (Fig. 9) and the time required for transcription to become maximal in the whole reaction (Fig. 8B). This suggests that the synthesis of 5S RNA is limited by the rate of the association of this factor with the stable transcription complex.

## DISCUSSION

Accurate transcription by all three classes of eucaryotic RNA polymerase has been shown to involve specific and stable associations between transcription factors and sequences in the promoter region of the gene (3, 6, 7, 25). It is on these stable transcription complexes that the RNA polymerases initiate synthesis. The experiments described in this paper demonstrate a selective insolubility of the stable complexes that form on cloned DNA templates in transcription reactions containing S-100 or whole-cell extracts. The activated templates and associated limiting transcription factors are selectively and quantitatively pelleted by a mild centrifugation. We showed that this centrifugal isolation is applicable to stable transcription complexes of all three classes of eucaryotic polymerase.

Centrifugal isolation of active templates provides a rapid and virtually quantitative means of purifying eucaryotic transcription factors. For instance, the rDNA transcription complex cosedimented with only 2 to 4% of the protein of the S-100 extract (Table 1). Thus, the limiting polymerase I

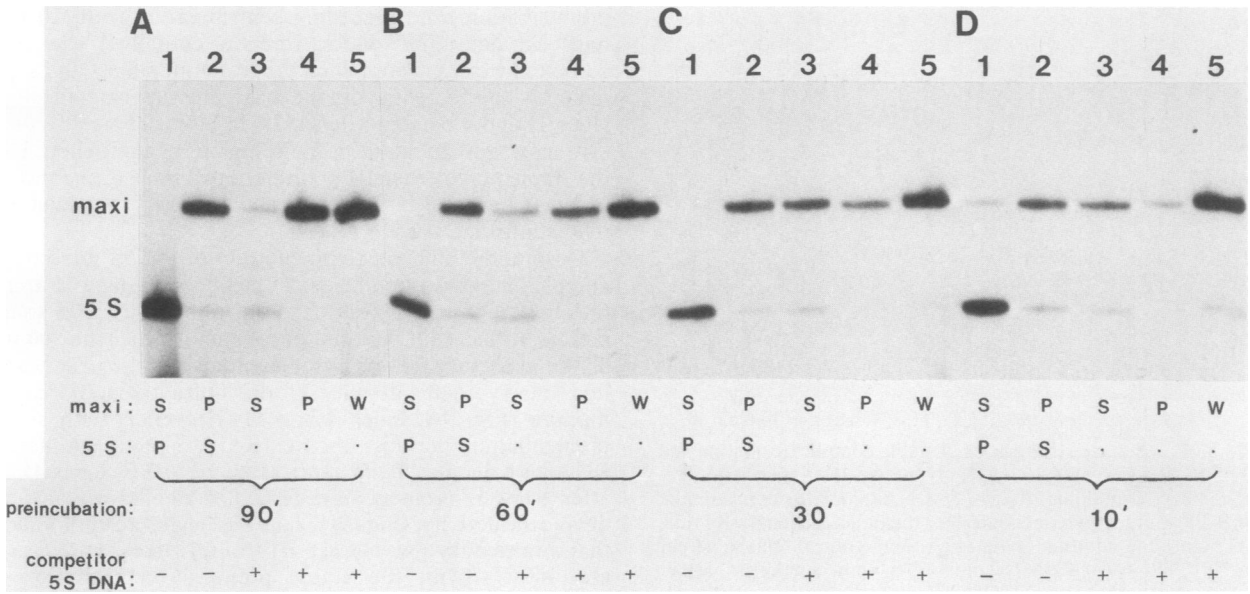


FIG. 9. Polymerase III: centrifugal fractionation of components required for 5S RNA synthesis. Supernatants and pellets were prepared from reaction mixtures containing S-100 extract and 10 μg of either the 5S or maxigene template per ml and preincubated for (panel A) 90 min, (panel B) 60 min, (panel C) 30 min, and (panel D) 10 min. The resultant fractions were assayed for transcriptional capacity. Lanes: 1, 5S gene pellet combined with maxigene supernatant; 2, maxigene pellet combined with 5S gene supernatant; 3, maxigene supernatant; 4, maxigene pellet suspended in buffer; 5, maxigene whole reaction mixture. Assays shown in lanes 3 through 5 were performed in the presence of 10 μg of 5S DNA competitor per ml. W, Whole reaction mixture; P, pellet; S, supernatant.

transcription factors are purified 25- to 50-fold by centrifugal isolation. Accurate rRNA synthesis requires at least two activities of the cell extract (a heat-sensitive component called factor C and species-specific factor D; 14, 16; Sollner-Webb et al., in press). In other studies, we complemented the supernatant and pellet fractions with C and D resolved by phosphocellulose chromatography and found that both of these factors were quantitatively pelleted on centrifugation of a transcription reaction mixture (J. Tower, V. Culotta, and B. Sollner-Webb, manuscript in preparation). Centrifugal isolation thus provides a favorable alternative to ion-exchange chromatography as an initial step in purifying transcription factors, since the traditional techniques are considerably more time consuming and generally result in significant losses of activity.

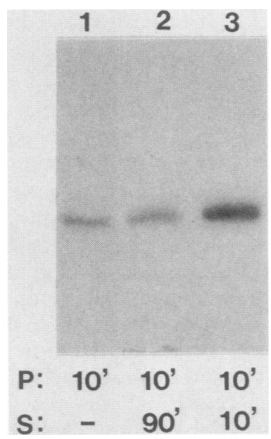


FIG. 10. Polymerase III: the slowly associating factor is absent from supernatants prepared after 90 min of preincubation. Pellets prepared as described in the legend to Fig. 8 from reaction mixtures preincubated for 10 min were suspended either in buffer (lane 1) or in supernatant derived from reaction mixtures preincubated for 90 (lane 2) or 10 (lane 3) min. Transcriptional capacity was measured as in the legends to Fig. 7 and 8. P, Pellet; S, supernatant.

It should be noted that only 10% of the polymerase I activity present in the S-100 extract cosedimented with the activated rDNA template (Table 1). This suggests that the reaction mixtures might contain a 10-fold excess of polymerase I. Alternatively, other experiments (Tower et al., in preparation) suggest that only the subset of polymerase I that cosediments with the active template is competent for accurate rRNA synthesis.

Rapid sedimentation of transcription complexes is a general phenomenon, but the transcriptional capacity of the isolated complex differs for the three polymerase classes (Table 2). Stable polymerase I complexes of both mouse and human rDNA formed in either S-100 or whole-cell extracts sedimented to the pellet and exhibited the full transcriptional activity of unfractionated reaction mixtures (Fig. 1 and 6). On the other hand, stable polymerase II preinitiation complexes formed on the promoter of the adenovirus major late transcription unit by incubation in a HeLa whole-cell extract also quantitatively sedimented, but these complexes lacked a necessary factor (Fig. 7). This additional component remained in the supernatant irrespective of the preincubation time, implying that an essential polymerase II transcription factor is not part of the stable adenovirus preinitiation complex.

Polymerase III studies involved transcription of a 5S RNA gene in the S-100 extract. Although the activated 5S DNA template was also found in the pellet when in vitro reaction mixtures were centrifuged, the transcriptional activity of this isolated preinitiation complex varied with the preincubation period (Fig. 9). When centrifuged after short preincubation periods, an essential transcription factor remained soluble, but after longer preincubation times, all of the components necessary for 5S RNA synthesis cosedimented. These re-

TABLE 2. Centrifugal fractionation of eucaryotic transcription complexes[a]

| RNA polymerase class | Fraction(s) needed for full activity |
|---|---|
| Polymerase I | P |
| Polymerase II | P + S |
| Polymerase III | P (90-min preincubation) |
| | P + S (10-min preincubation) |

[a] The partitioning of the stable complex and the components required for full transcriptional activity are summarized for reaction mixtures involving polymerase I, II and III. The stable complex was found in the pellet in all three cases. P = Pellet, P + S, pellet suspended in supernatant.

sults can be readily interpreted in terms of the known kinetics of complex formation on 5S DNA. Transcription factors TFIIIA and C rapidly form a stable complex on the template, but 5S RNA synthesis is limited by the slow binding of transcription factor B and polymerase III (11). We infer that after short preincubation periods, factor B, polymerase III, or both remain soluble and the pellet contains 5S DNA complexed with factors A and C, but after prolonged incubation all of the essential factors cosediment with the active DNA. Thus, centrifugal fractionation at the appropriate times should allow convenient isolation of 5S DNA stable complexes either containing or lacking the slowly associating components.

Although the exact mechanism of pelleting is not yet known, several relevant observations have been made. Figures 2, 3, and 4 demonstrate that specific pelleting of polymerase I transcription factors and rDNA molecules involves formation of the stable complex. The transcription factors were inherently soluble in the transcription buffer (Fig. 2); they also remained soluble in the presence of nonspecific DNA but were found in the pellet when complexed with template DNA (Fig. 3). In addition, active template molecules quantitatively precipitated under conditions in which the bulk of the input rDNA, which was transcriptionally inactive, remained soluble (Fig. 4). However, the pelleted stable complex evidently consists of more than a DNA molecule with its stably associated transcription factors, for the apparent sedimentation coefficient of the transcription complex formed on the excised 470-base-pair rDNA template (Fig. 4) was larger than 2,000S; this indicates that the stable complexes in these in vitro reaction mixtures are part of a large aggregate or precipitate. Ackerman et al. (1) similarly found that an adenovirus polymerase II transcription complex precipitates from solution. However, other investigators have reported stable complexes that do not precipitate from certain in vitro reaction mixtures. For instance, Tolunay et al. (24) showed that a polymerase II transcription complex formed on adenovirus DNA can be isolated as a ~60S structure. A similar sedimentation coefficient was found for a polymerase III transcription complex on a 5S RNA gene (Wingender et al. [27]), but these investigators also reported that some of their complexes were in an insoluble precipitate. Possibly, transcription complexes are not inherently insoluble, but under appropriate conditions they selectively form complexes with extract components that confer their rapid sedimentation. Finally, we wish to emphasize that the sedimentation of stable complexes reported here is selective for transcriptionally active genes and is not merely due to a nonspecific precipitation of DNA.

The in vivo structural organization of active genes is distinct from that of inactive chromatin. Transcribing cellular genes are closely associated with large sedimentable complexes known as the nuclear matrix (18, 19), and active endogenous rRNA genes are sequestered in large nucleolar particles. Moreover, the chromatin of active rRNA genes and active immunoglobulin genes is specifically pelleted after mild nuclease treatment (8, 20). The sedimentable stable complexes formed on cloned DNA in vitro may well reflect this structural organization of active cellular genes and should thereby facilitate analysis of these cellular associations. Alternatively, it is possible that the selective sedimentation of transcription complexes in vitro might reflect a fortuitous association of stable complexes with precipitable material. Even if this were the case, centrifugal fractionation is nonetheless a simple and powerful method for the purification of transcription factors and for the analysis of stable complex formation with all three classes of eucaryotic RNA polymerase.

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