Factor C*, the Specific Initiation Component of the Mouse RNA Polymerase I Holoenzyme, Is Inactivated Early in the Transcription Process

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Factor C* is the component of the RNA polymerase I holoenzyme (factor C) that allows specific transcriptional initiation on a factor D (SL1)- and UBF-activated rDNA gene promoter. The in vitro transcriptional capacity of a preincubated rDNA promoter complex becomes exhausted very rapidly upon initiation of transcription. This is due to the rapid depletion of C* activity. In contrast, C* activity is not unstable in the absence of transcription, even in the presence of nucleoside triphosphates (NTPs). By using 3'dNTPs to specifically halt elongation, C* is seen to remain active through transcription complex assembly, initiation, and the first ~37 nucleotides of elongation, but it is inactivated before synthesis proceeds beyond ~40 nucleotides. When elongation is halted before this critical distance, the C* remains active and on that template complex, greatly extending the kinetics of transcription and generating manyfold more transcripts than would have been synthesized if elongation had proceeded past the critical distance where C* is inactivated. In complementary in vivo analysis under conditions where C* activity is not replenished, C* activity becomes depleted from cells, but this also occurs only when there is ongoing rDNA transcription. Thus, both in vitro and in vivo, the specific initiation-conferring component of the RNA polymerase I holoenzyme is used stoichiometrically in the transcription process.

The rRNA genes (rDNA) are transcribed in the nucleolus by RNA polymerase I and account for about half of the RNA synthesis of a growing mammalian cell (reviewed in references 57 and 58). Specific transcription of rDNA requires a component that has been called factor D (43, 60), TFID (9), TIF-IB (52), SL1 (34, 56), or Rib-1 (40) and consists of the TATA-binding protein plus three polymerase I-specific associated factors (13). A second rDNA transcription factor, UBF, binds DNA through multiple regions with homology to high-mobility-group (HMG) proteins, and its observed roles range from being essential for in vitro rDNA transcription (40, 53) to being stimulatory (3, 35, 56) to having no effect on transcription (56), depending on the assay conditions and possibly also on the species examined. UBF may act by binding to the rDNA promoter (2, 27, 64), by augmenting the binding of factor D to the promoter (2), and/or by preventing the action of inhibitory components (30, 31). Together, factor D and UBF assemble the rDNA promoter into a stable complex that can direct more than 20 rounds of transcription (23).

The factor D/UBF-activated rDNA promoter complex is then recognized by a specific initiation-competent form of RNA polymerase I, factor C. Factor C is distinct from the bulk of isolated polymerase I, which can elongate and initiate nonspecifically but cannot by itself initiate selectively on a factor D/UBF-activated rDNA promoter. Factor C activity copurifies with bulk RNA polymerase I through biochemical purification of the characteristic polymerase I polypeptides and through gradient elution from four additional chromatographic matrices, and it is precipitated by multiple anti-

polymerase I antibodies (61). Factor C remains intact through considerably more stringent chromatographic conditions than are used to dissociate the specific initiation competence-conferring sigma subunit from the prokaryotic RNA polymerase holoenzyme (5). However, components that convey specific initiation competence to RNA polymerase I have now been separated from the RNA polymerase I elongation activity and are variously called C* (this study), TFIC (38), and TIF-IA (54). These three activities, isolated by different methods, may well represent the same biochemical component, but this remains to be verified.

Cellular rDNA transcription is closely regulated according to the growth state of the cell. It is substantially down-regulated upon protein synthesis inhibition (e.g., cycloheximide and puromycin treatment and amino acid starvation), quiescence, and treatment with certain hormones and viruses (6, 8, 50; see references 19, 42, and 45 for reviews), and it is comparably up-regulated upon resumption of protein synthesis, upon replenishment of serum, and in particular cases of increased protein synthesis (21, 63). From the rapid decrease in rDNA transcription upon inhibition of cellular protein synthesis, it has long been inferred that rDNA transcription requires a short-lived component that needs ongoing protein synthesis for replenishment (12, 33, 44, 46, 59). Mirroring these in vivo phenomena, extracts prepared from cells inhibited for protein synthesis by various treatments, from stationary-phase cells, from hormone-treated cells, and from poliovirus-infected cells have correspondingly diminished capacities to initiate rDNA transcription in vitro, and in each case this is due to their being severely limited for factor C (TIF-IA, TFIC) activity (6, 7, 16, 60, 61); the bulk polymerase I activity is not reduced relative to control cell extracts. These results would imply that factor C is the earlier inferred short-lived component that is central to rDNA transcriptional regulation.

The experiments described in this report demonstrate that

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RNA synthesis from a preformed rDNA transcription complex is completed very rapidly and that this is due to the stoichiometric consumption of factor C* in the rDNA transcription process. Addition of isolated factor C* restores transcription to the exhausted reaction. C* activity is specifically inactivated between ~37 and ~40 nucleotides (nt) of elongation; it is not inherently unstable, nor is it simply dissociated from the polymerase or sequestered on the transcription complex. When transcribing an RNA product of <34 nt, the reaction yields manyfold more RNA molecules, continues for much longer, and does not become exhausted of C* activity. Our *in vitro* results mirror the *in vivo* situation in which a polymerase I transcription component that was originally thought to be inherently short-lived turns out to be factor C* and to be consumed by the cellular rDNA transcription process.

MATERIALS AND METHODS

Extracts. S-100 extracts, used for preinitiation complex formation, were prepared from log-phase mouse L1210 cells as described previously (41), followed by precipitation with 60% saturated ammonium sulfate to remove nucleoside triphosphates (NTPs). The precipitation solution was rocked for 30 min and centrifuged at $15,000 \times g$ for 15 min, and the pelleted transcription components were resuspended in the original cell lysate volume of dialysis buffer (20% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES; pH 7.9], 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 2 μ M leupeptin, 0.5 μ M pepstatin A) at 100 mM KCl and dialyzed for 5 h. All treatments were at ~4°C unless otherwise specified.

Cycloheximide-treated cell extracts, used for complementation assays of factor C*, were prepared from log-phase L1210 cells that had been treated with 0.1 mg of cycloheximide per ml for 120 min prior to S-100 extract preparation. The stationary-phase cell S-100 extract, also used for the C* complementation assays, was prepared from L1210 cells that had grown into stationary phase (61).

For the S-100 extracts in Fig. 7, log-phase L1210 cells were (i) untreated, (ii) treated with 0.3 μ g of actinomycin D per ml for 120 min, (iii) treated with 0.1 mg of cycloheximide per ml or 0.25 mg of puromycin per ml for 90 min, or (iv) treated both with 0.3 μ g of actinomycin D per ml for 120 min and with 0.1 mg of cycloheximide per ml or 0.25 mg of puromycin per ml for the last 90 min of the 120 min. High-salt S-100 extracts were prepared from these cultures as described previously (41), except that the 15-min extraction, with rocking, was with 0.38 volume of 4 M KCl plus 0.15 volume of 300 mM HEPES (pH 7.9)–30 mM $MgCl_2$ (25), and after centrifugation the transcription components were precipitated with 45% ammonium sulfate and dissolved and dialyzed as described above.

Fractionation. Factor C and factor D plus UBF (containing no detectable C* or polymerase activity) were prepared by chromatography of S-100 extract on phosphocellulose P-11, eluted between 0.35 and 0.6 M KCl and between 0.8 and 1.2 M KCl, respectively, and dialyzed to 0.1 M KCl (60). Factor C* was isolated from 160 ml of a mouse Ehrlich ascites cell nuclear extract (61) depleted of factor D activity by passage through a 100-ml Bio Rex 70 column at 0.7 M KCl. (This and all following solutions were in dialysis buffer, brought to the indicated KCl concentration, except that the P-11 and Mono Q buffers lack leupeptin and pepstatin A.) Following dialysis to 0.1 M KCl, the solution was loaded onto a 200-ml DEAE-Sephacel column and washed in 0.1 M KCl. The 0.1 to 0.26 M KCl eluate (40 ml) was loaded onto a 40-ml phosphocellulose P-11 column. After being washed with 100 ml of 0.35 M KCl

and 50 ml of 0.6 M KCl, the 0.6 to 0.8 M KCl eluate (45 ml) containing some of the factor C activity was diluted to 0.1 M KCl and loaded onto a 1-ml Mono Q column (Pharmacia). The Mono Q column was eluted with a 16-ml linear gradient from 0.1 to 0.35 M KCl, followed by 8 ml of 0.325 M KCl and then a 14-ml linear gradient from 0.325 to 0.6 M KCl. RNA polymerase I elutes just before 0.325 M KCl, and factor C* elutes at ~0.38 M KCl. C* and polymerase I were dialyzed against 0.1 M KCl in dialysis buffer.

Transcription reactions. All preincubation and transcription reaction mixtures contained 20 mM HEPES (pH 7.9), 90 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 300 μ g of α -amanitin per ml in a final volume of 25 μ l, unless otherwise specified, and were incubated at 30°C. Transcripts were isolated and electrophoretically analyzed as described previously (41). Autoradiograms used for quantitation were densitometrically scanned.

Templates. Mouse p5'Sal-Pvu (41) was truncated with *Tth*111I at residue +450 (in Fig. 1 and 3) or with *Nde*I at residue +520 (in Fig. 2, 3, and 7). The mouse closed-circular template 5' Δ -163 was irradiated with increasing doses of UV (23) for the experiment in Fig. 4A. The mouse super promoter (S) template is a -36/-28(-5) linker-scanning deletion mutant of the *Xenopus laevis* rDNA promoter (47), and the S/v template has the -245 to +16 region of the S template subcloned between the *Sal*I and *Sma*I sites of pUC18, so that the transcript beyond residue 16 derives from pUC vector sequences (see Fig. 4B and C, 5, and 6).

C* assay in extracts. Factor C* was assayed for its ability to stimulate transcription in a reaction mixture containing 5 μ l of cycloheximide-treated cell extract; 7 μ g of template per ml; 500 μ M each ATP, GTP, and UTP; and 50 μ M [α - 32 P]CTP (3 μ Ci) incubated for 45 min. In the C* assays of Fig. 2, lanes 1 and 2 and lanes 8 to 12, stationary-phase cell extract or isolated fractions, respectively, were used in place of the cycloheximide-treated cell extract.

Time course reactions. Reaction mixtures containing 1.5 μ l of a log-phase cell extract, 5.5 μ l of a cycloheximide-treated cell extract (to ensure that C* is limiting), and 7 μ g of the indicated template per ml were preincubated at 30°C in the absence of NTPs for the indicated periods. Then 500 μ M each ATP, GTP, and UTP and 50 μ M CTP (final concentrations) were added, and incubation was continued for the indicated times. Finally, 3 μ Ci of [α - 32 P]CTP was added, and the incubations were continued for an additional 5 min before being terminated (see Fig. 1B). In Fig. 1A, the preincubation without NTPs was omitted.

Reactions to assay C* following an initial transcription period. Preinitiation complexes assembled in 25- μ l (in Fig. 6) or 40- μ l (in Fig. 3 to 5) reaction mixtures containing 4 μ l of extract depleted of NTPs (see above) plus 3 μ g of template per ml were incubated at 30°C for 60 min and then separated from any residual unbound factors by pelleting at $10,000 \times g$ for 5 min at 4°C and resuspension in 25 μ l of transcription buffer lacking NTPs (14). For the time course reactions, resuspended preinitiation complexes formed on the 450-nt runoff template were supplemented with 500 μ M each ATP, GTP, and UTP and 50 μ M CTP (in Fig. 3A and C) or were not supplemented (in Fig. 3B) and incubated at 30°C for the indicated periods. For the UV-treated template (in Fig. 4A), the resuspended preinitiation complexes were treated as above with a 45-min incubation period with the NTPs. For reactions involving 3'dNTPs (in Fig. 4 to 6), the resuspended preinitiation complexes were incubated for 45 min following addition of no NTPs, of 500 μ M ATP and UTP plus 50 μ M GTP and 3'dCTP (to stop elongation at C residues), of 500 μ M ATP and CTP

FIG. 1. Time course of rDNA transcription in vitro. (A) Reaction mixtures containing a 450-nt runoff template, extract, and unlabeled NTPs were immediately placed at 30°C and allowed to transcribe for the indicated periods before [α - 32 P]CTP was added for a 5-min labeling period. (B) Reaction mixtures as in panel A, except lacking the NTPs, were preincubated for 1, 2, or 3 h, as indicated. Unlabeled NTPs were then added, and the reactions were allowed to transcribe for the indicated times before [α - 32 P]CTP was added for a 5-min labeling period. Lanes 19 to 22 were incubated in the absence of NTPs for 1 h and in the presence of NTPs for 30 min, and then the designated transcription factor(s) and [α - 32 P]CTP were added for a 10-min labeling period. These added factors do not contribute NTPs.

plus 50 μ M GTP and 3'dUTP (to stop elongation at U residues) along with the indicated concentrations of UTP, or of 500 μ M ATP, CTP, and UTP plus 50 μ M GTP (to allow elongation). (To visualize the short transcripts formed in these 3'dNTP-terminated reactions, 12 μ Ci of [α - 32 P]GTP was added along with the NTPs for a 45-min transcription period [in Fig. 4C and 5B] or after the indicated times for a 5-min labeling period [in Fig. 6], and then the RNA was isolated and resolved on a 20% polyacrylamide gel.) The number of transcripts of each length was determined from the intensities of the transcript bands of that size (measured by densitometry) divided by the number of G residues in that transcript (calculated from the nucleotide sequence of the template).

To assay remaining C* activity, the unlabeled reactions prepared as described in the previous paragraph were put on ice and the transcription complex was pelleted by centrifugation at 10,000 $\times$ g for 5 min at 4°C (14). After careful removal of the supernatants, transcription factors bound to the template were released by micrococcal nuclease digestion in a 4- μ l reaction mixture (60) (in Fig. 3 to 5) or by high-salt treatment (in Fig. 3C; pellets suspended in 2 μ l of 1 M KCl and incubated at 22°C for 5 min), and the nuclease was inactivated by addition of 1.5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Finally, the C* activity of the pellet or supernatant fraction or both was assayed by adding to a reaction mixture containing 1.5 μ l of a cycloheximide-treated cell extract, 7 μ g of the 520-nt runoff template per ml, 0.8 U of RNasin per μ l (Promega Biotec), and either 500 μ M ATP, GTP, and UTP plus 50 μ M [α - 32 P]CTP (3 μ Ci) (in Fig. 3 and 4A) or 500 μ M ATP, CTP, and UTP plus 50 μ M of [α - 32 P]GTP (3 μ Ci) (in Fig. 4B and 5A). The reaction mixtures were incubated at 30°C for 45 min, and RNA was isolated and analyzed as described previously (41). In all cases, the pellet, the supernatant, and the supernatant plus pellet were assayed. The supernatant was reproducibly inactive, as in Fig. 3, and the supernatant plus pellet showed no more activity than the pellet alone.

RESULTS

Kinetics of in vitro rDNA transcription. In our typical in vitro rDNA transcription reaction in which factor C* is limiting (see Discussion), when transcription is initiated immediately upon addition of rDNA template to the mouse S-100 extract, rRNA is synthesized at a fairly constant rate for over 30 min (Fig. 1A). In contrast, when the template and extract are preincubated for 1 h prior to addition of the NTPs, the transcription shows strikingly rapid kinetics, with virtually all synthesis taking place within the first few minutes (Fig. 1B, lanes 1 to 6). Despite the very different kinetics, the total amount of RNA synthesis that takes place in the reaction that was not preincubated is approximately the same as in the one that was preincubated.

We examined the basis of the rapid cessation of synthesis in the preincubated reaction. It is not caused by incubation of the reaction per se, because an additional preincubation of 1 or 2 h does not substantially alter the extent or kinetics of synthesis following NTP addition (Fig. 1B, lanes 7 to 18). Neither is it due to the presence of NTPs, since preincubation with all four NTPs under conditions that inhibit elongation does not reduce the amount of resultant transcription (in Fig. 4B and 5A; data not shown). The rapid cessation of transcription also does not result from a destruction of the NTPs (Fig. 1B, lanes 19 and 20). Rather, it appears due to the process of rDNA transcription itself, as if a factor in the extract can catalyze only a fixed amount of transcription, and once that has taken place, synthesis stops.

To determine what limits the amount of transcription, the individual rDNA transcription factors were added to a reaction whose synthetic capacity had been exhausted by a 30-min transcription period following a 1-h preincubation in the absence of NTPs. Addition of the promoter-binding factors D and UBF to such an exhausted reaction (like that of Fig. 1B, lane 6) did not restore transcription (lane 22). In contrast, transcription was restored when the exhausted reaction was

FIG. 2. Both factor C* and RNA polymerase I are required for rDNA transcription in vitro. The factors indicated below the lanes, prepared as described in Materials and Methods, were added to transcription reaction mixtures containing NTPs, [α - 32 P]CTP, a 520-nt runoff template plus extract of stationary-phase (Stat.) cells (lanes 1 and 2), extract of cycloheximide-treated (Cyclo.) cells (lanes 3 to 7), or the isolated factors D and UBF (lanes 8 to 12). The RNA products were analyzed after a 45-min transcription period. Comparable amounts of RNA polymerase I elongation activity were added to the reaction mixtures in lanes 5, 6, 9, 11, and 12. Severalfold more C* activity was added to the reaction mixture in lane 2 than to the others.

supplemented with factor C, the initiation-competent form of RNA polymerase I (lane 19). To further delineate the activating component, factor C was dissociated into pol (an elongation-competent but specific initiation-deficient RNA polymerase I) and factor C* (which provides the specific initiation capacity to pol but lacks polymerase activity itself [see below]). Addition of factor C* restored transcription to the exhausted reaction (lane 20), while addition of pol did not (lane 21). Thus, factor C* is the component that is consumed during rDNA transcription.

Separation of factor C* from bulk RNA polymerase I. Although factor C had previously been found to behave as one entity through purification to the characteristic complement of RNA polymerase I polypeptides (61), passage of a factor C preparation over Mono Q yields a factor C* that restores specific rDNA transcription to extracts of stationary-phase and cycloheximide-treated cells (Fig. 2, lanes 1 to 3 and 7) yet contains no detectable RNA polymerase I activity (data not shown). The Mono Q fractionation also yields a C*-deficient RNA polymerase I fraction (pol) that does not restore transcription to the inactive extract (lane 6). When used in combination with factors D and UBF, specific transcriptional initiation does not occur unless factors C* and pol are both present (lane 10 to 12). This confirms that factor C can be separated into a bulk RNA polymerase I and a specific initiation-competence factor C* and that both are needed for rDNA transcription. These separated activities were used to identify factor C* as the component that becomes exhausted by in vitro transcription (Fig. 1B). Furthermore, since rDNA transcription is restored in extracts of cycloheximide-treated cells only by addition of C* and in a dose-dependent manner (Fig. 2 and data not shown), C* activity can conveniently be assayed by supplementing a cycloheximide-treated cell extract; this assay is used in the following experiments.

Analysis of factor C* loss in vitro. To further study the rapid loss of available factor C* upon transcription (Fig. 1) and to examine whether factor C* became limiting as a result of inactivation or of sequestration on the transcription complex in a manner that prevents reinitiation, we released factors from the transcription complex and assayed the resultant C* activity (Fig. 3). For this, we made use of earlier observations that the rDNA transcription complex can readily be sedimented and that all factors necessary for rDNA transcription can then be

FIG. 3. Effect of transcription on C* activity assayed by factor release. Preinitiation complexes recovered after a 1-h incubation of extract with a 450-nt runoff template in the absence of NTPs (as described in Materials and Methods) were incubated at 30°C in the presence (A) or absence (B) of added NTPs for the indicated times. Reaction mixtures were chilled on ice and centrifuged to sediment the template and bound factors. Factors were released from the pelleted complex by digestion with micrococcal nuclease (MNase) and subsequent nuclease inactivation with EGTA (panels A and B; panel C, lanes 1 and 2) or by resuspension in 1 M KCl (panel C, lanes 3 to 6). In panel A, lane 13, the micrococcal nuclease was inactivated with EGTA prior to its addition to the pellet. Pellets and supernatants were assayed for C* activity by the ability to complement a cycloheximide-treated cell extract in the transcription of a 520-nt runoff during a 45-min incubation as described in Materials and Methods. In panels A and B, pellets and supernatants were assayed separately. In panel C, pellets and supernatants (Sup) were mixed and assayed.

released in an active form by treating the recovered complex with micrococcal nuclease or with high salt (14, 60). The factor C* from the pelleted complex, from the supernatant, and from the combined pellet plus supernatant are then assayed by addition to cycloheximide-treated cell extract along with a second rDNA template.

Preincubated reactions were allowed to transcribe for various periods, and the remaining C* activity was determined after release by micrococcal nuclease or KCl treatment, as indicated (Fig. 3). C* activity of the template complex assayed in this manner displays the same rapid consumption (Fig. 3A, lanes 1 to 6) and is transcription dependent (Fig. 3B, lanes 1 to 6), like the loss of synthetic capacity of the transcribing extract (Fig. 1B). This implies that C* activity is not inactivated as a result of sequestration on the transcription complex. Active C* is also not released to the supernatant upon transcription (Fig. 3A, lanes 7 to 12). Neither is C* significantly detected in the supernatant following a micrococcal nuclease treatment that degrades the released transcripts without leaving perceptible nuclease-resistant RNA cores (data not shown). Furthermore, C* activity is also not reconstituted by combining the released pellet and supernatant fractions (Fig. 3C). Thus, the loss of C* activity upon transcription is not due to its sequestration on the

FIG. 4. Effect of transcript length on C^* activity. (A) Preinitiation complexes were recovered after a 1-h incubation of extract with a closed-circular template that had been UV irradiated for various times to produce average transcript lengths of 0.3 kb (lane 2), 0.5 kb (lane 3), or 1 kb (lane 4) or the same template that had not been UV irradiated and produced a transcript length of >3 kb (lanes 1 and 5), as described in Materials and Methods. They were then incubated for 45 min in the presence (lanes 2 to 5) or absence (lane 1) of NTPs. The remaining factor C^* was then recovered and assayed as in Fig. 3A, lanes 1 to 6 (see Materials and Methods). (B) Preincubation and initial transcription reactions were performed, and the remaining C^* activity was assayed as in panel A, except that the preinitiation complexes were formed on the S (lanes 2 and 4) or S/v (lanes 1, 3, and 5) template and the reaction mixture for the 45-min initial transcription reaction contained 50 μ M 3'dCTP (lane 2) or 3'dUTP (lanes 3 and 4) and the other three normal NTPs. In lane 1, no NTPs were added, and lane 5 contained all four normal NTPs. (C) Preincubation and initial transcription reactions were performed identically to those of panel B, except that [α - 32 P]GTP was included during the 45-min initial transcription period, after which the RNA was isolated and analyzed. The lane numbers correspond to those of panel B. The marker lane, M, shows the migration of a 30-nt runoff transcript from an S/v template linearized with *Eco*RI. The ethanol precipitation of the 10-nt RNA in lane 2 was probably not quantitative. The two panels are printed to different exposures.

transcription complex or to dissociation in a manner that can reconstitute in our *in vitro* reaction.

The control in Fig. 3A (lane 13) confirms that C^* stably binds the preinitiation complex and does not release to initiate on a second template on its own. This experiment is analogous to that of lane 1, except that the nuclease digestion was inhibited, in which case C^* participates in transcription of the first template but does not transfer to the second template, which had been assembled with the C^* -deficient extract.

To determine the point in the transcription process at which C^* activity is lost, we prematurely terminated elongation at various positions and assayed C^* as in Fig. 3A, lanes 1 to 6. When the initial transcription occurred on templates that had been UV irradiated to halt elongation after an average of 0.3, 0.5, 1, or >3 kb, C^* activity was lost (Fig. 4A, lanes 2 to 5) relative to a control reaction in which no initiation took place (lane 1). The recovery of very low levels of C^* activity following transcription on the most-irradiated template (lane 2) suggests that loss of C^* activity may be correlated with the length of the transcript produced; a small fraction of the template molecules

having UV-induced lesions close to the initiation site could have halted transcription while C^* was still active.

The elongation process was then stopped at known locations closer to the initiation site by transcribing various templates with three normal NTPs and one 3'dNTP. By using the S template (47), we found that transcription stopped at 10 and 16 nt with 3'dCTP and at 54 nt with 3'dUTP (Fig. 4C, lanes 2 and 4); when this promoter was used cloned into pUC at residue +16 (S/v), transcription stopped at 20, 28, and 33 nt with 3'dUTP (Fig. 4C, lane 3). (Residual amounts of CTP and UTP allow some readthrough of the first expected stop.) The remaining C^* activity was then assayed (Fig. 3B [reactions carried out as for Fig. 3A]). Full C^* activity (Fig. 3B, lane 1) was recovered when transcription extended 10 and 16 nt (lane 2) or 20, 28, and 33 nt (lane 3). However, when transcription extended 54 nt (lane 4) or around the entire plasmid (lane 5), no C^* activity was recovered. Therefore, C^* remains active when the polymerase has transcribed up to 33 nt, but its activity is lost at or before 54 nt.

To confirm that loss of C^* activity is dependent on the length

FIG. 5. C* inactivation after transcription of 37 to 40 nt. (A) Preinitiation and initial transcription reactions were performed as in Fig. 4B with the S/v template, but the 3'dUTP of the initial transcription period was diluted with the indicated concentrations of UTP. Remaining C* activity was assayed as in Fig. 4B and quantitated by densitometric scanning of the autoradiogram. (B) Reactions were set up identically to those of panel A, except that [α - 32 P]GTP was included during the 45-min initial transcription period, after which RNA was isolated and analyzed. Lanes 1 to 6 correspond to those of panel A. Lane 7 shows an identical reaction with the S template and 3'dUTP, generating the 54-nt transcript. The number of transcripts of ≥ 40 and ≥ 37 nt, relative to the number of transcripts of that size in lane 7, was determined from densitometric scans, taking into account the [α - 32 P]G content of each size of RNA.

of the resultant transcript, reactions with the S/v template and 3'dUTP were supplemented with increasing concentrations of UTP to allow a controlled elongation to U residues beyond position 33 in the transcript (Fig. 5B). In contrast to the full C* activity that is recovered when no UTP is added and transcription extends to 20, 28, and 33 nt (Fig. 5A, lanes 0 and 1, and 5B, lane 1), when the transcripts become longer as a result of the increasing UTP concentrations (Fig. 5B), progressively less C* activity is retained (Fig. 5A).

The critical length of the transcript at which C* activity is lost can be calculated from the data in Fig. 5. When a transcript extends to or beyond this critical length, the C* of that transcription complex will be inactivated, as is observed when transcription extends to 54 nt on the S template (Fig. 4). The fraction of C* lost at each UTP concentration with the S/v template (Fig. 5A) should therefore equal the number of transcripts that are equal to or longer than the critical length (determined from Fig. 5B [see Materials and Methods]), relative to the number of 54-nt transcripts obtained in a parallel reaction with the S template and no added UTP (Fig. 5B, lane 7). With 5 μ M added UTP, 64% of C* activity is lost (Fig. 5A, lane 3) and there are 63% as many transcripts of ≥ 40 nt (Fig. 5B, lane 3) as in the control reaction (lane 7). Similarly, with 10 μ M UTP, 71% of C* activity is lost (Fig. 5A, lane 4)

and there are 69% as many transcripts of ≥ 40 nt (Fig. 5B, lane 4) as in the control reaction (lane 7). These values, when calculated for transcripts of ≥ 37 nt, correlate less well (Fig. 5). These results were confirmed by similar analysis of a parallel experiment in which the C* was instead released by high-salt treatment (data not shown), indicating that C* activity is lost when transcription proceeds between 37 and 40 nt from the start site.

Recycling of C* in prematurely terminated reactions. Since C* remains active following the synthesis of the 20-, 28-, and 33-nt transcripts, might that C* be reused to form more than one such short RNA molecule? In multiple experiments with several extracts, we have observed from 4 to more than 10 times as many 20-, 28-, and 33-nt transcripts as 54-nt transcripts produced in the respective 3'dUTP-halted reactions (e.g., Fig. 4C, lane 3 versus 4, and Fig. 5B, lane 1 versus 7; note that the longer transcripts contain more radiolabeled residues and therefore appear relatively more intense than the shorter ones). Consistent with obtaining multiple 20-, 28-, and 33-nt transcripts per active template complex, sedimentation experiments show that all our 3'dUTP-terminated transcripts are released from the template complex since they are recovered in the supernatant fraction after a pelleting step (data not shown). These combined data indicate that when transcription

FIG. 6. Reinitiation by factor C* when elongation is halted before C* inactivation. Preinitiation complexes were formed as in Fig. 4C, with the S/v (A) or S (B and C) templates; the reaction mixtures in panel C contained an additional 1.5 μ l of partially purified factor C*. The recovered preinitiation complexes were brought to 500 μ M ATP and CTP, 25 μ M GTP, and 50 μ M 3'dUTP and allowed to transcribe for the indicated times before [α - 32 P]GTP was added for a 5-min labeling period.

is stopped at ≤ 33 nt with 3'dUTP, the RNA is released from the template and the C* can be reused to catalyze additional rounds of synthesis.

Additional studies show that when the S template (that generates the 54-nt transcripts with 3'dUTP) was preincubated with all the transcription components except C* and then added to a 3'dUTP-containing transcription reaction in which the S/v template is recycling to synthesize multiple 20-, 28-, and 33-nt transcripts, virtually no 54-nt transcripts are generated (data not shown). This indicates that the C* does not transfer from the S/v to the S template, even though it does recycle on the S/v template. This, in turn, suggests that the C* recycling on templates where elongation is halted at less than 40 nt does not involve a complete release and rebinding of the C*/polymerase; possibly these factors still remain in an "initiation mode" contacting factor D/UBF at the promoter.

From the observations that (i) transcription is completed very rapidly in the preincubated reaction as a result of C* inactivation (Fig. 1) and (ii) C* remains active and can recycle when synthesis is stopped at or before 33 nt (Fig. 4), one might anticipate that transcription in a reaction producing short RNAs would have a considerably extended time course. This was tested by using the S and S/v templates in the presence of 3'dUTP. The reaction that generates the 54-nt RNA exhibits rapid kinetics (Fig. 6B), like the reaction that generates a long runoff RNA (Fig. 1B), and the reaction that generates the 20-, 28-, and 33-nt RNAs has about the same initial rate of synthesis (Fig. 6A). However, transcription of the 20-, 28-, and 33-nt RNAs can continue at this rate for over 30 min (Fig. 6A). This is consistent with factor C* finishing transcription of one

short RNA and then recycling to transcribe additional short RNAs on that template complex.

Would a preincubated transcription reaction also exhibit extended kinetics of synthesis of a longer RNA if factor C* were in excess during the preincubation? Figure 6C shows that such an extended synthesis period can indeed be induced when additional factor C* is present during the preincubation of a reaction set up otherwise like that of Fig. 6B. This illustrates that the rapid exhaustion of C* activity is not readily apparent in a reaction that contains excess C*. Indeed, extracts prepared from actinomycin D-treated cells, which have more C* activity than do extracts of untreated cells (61), also show protracted kinetics of synthesis in a preincubated reaction (13a).

In vivo inhibition of rDNA transcription similarly prevents loss of C* activity. C* activity is known to be lost from cells when cellular protein synthesis is inhibited (16, 54, 60, 61). We wondered whether this in vivo loss of C* activity might be equivalent to the rapid loss of C* activity that occurs during in vitro rDNA transcription (Fig. 1), since in both cases there is rDNA transcription and no protein synthesis and C* activity becomes depleted. This would imply that in an untreated growing cell, C* activity would be continually lost as a result of ongoing rDNA transcription but would be continually replenished in a protein synthesis-dependent manner. In this scenario, the net loss of cellular C* activity observed when cellular protein synthesis is inhibited would require that there be ongoing rDNA transcription. (Without rDNA transcription to consume the C*, a pool of active C* would remain, even though C* replenishment is inhibited by blocking protein synthesis.)

FIG. 7. C* activity from cells treated with inhibitors of protein synthesis and rDNA transcription. Log-phase L1210 cells were supplemented with 0.3 μ g of actinomycin D per ml to selectively inhibit rRNA synthesis (lanes 3 and 4) or were not supplemented (lanes 1 and 2). Thirty minutes later, they were supplemented with 0.1 mg of cycloheximide per ml (panel A, lanes 2 and 4) or 0.25 mg of puromycin per ml (panel B, lanes 2 and 4) to inhibit protein synthesis, or were not supplemented (lanes 1 and 3). These conditions are indicated below the individual lanes. Ninety minutes later, S-100 extracts were prepared, and their C* activity was assayed by adding 1 μ l to a transcription reaction mixture containing 5 μ l of an extract of cycloheximide-treated cells and the 520-nt runoff template, initiating a 45-min synthesis period.

Figure 7, lanes 1 and 2, confirms that C* activity is markedly diminished in cells whose protein synthesis has been inhibited by treatment with cycloheximide (which halts ribosome progression) or with puromycin (which prematurely releases nascent polypeptides), relative to the C* activity from untreated control cells. Treatment with 0.3 μ g of actinomycin D per ml has been shown to selectively inhibit cellular rDNA transcription while not substantially depressing total RNA polymerase II transcription (reference 49 and data not shown). Strikingly, in such actinomycin D-treated cells, when protein synthesis is inhibited, factor C* activity is retained (Fig. 7, lanes 4). This implies that *in vivo*, as *in vitro*, C* activity is not inherently unstable but instead becomes depleted as part of the rDNA transcription process. When rDNA transcription is blocked, C* activity remains stable (Fig. 7, lanes 3 and 4). However, when C* activity is not replenished, as evidently occurs both in the protein synthesis-inhibited cell and in the mouse *in vitro* transcription reaction, the rDNA transcription-dependent depletion of C* results in a net loss of C* activity.

DISCUSSION

Factor C* is inactivated early in the elongation process. Transcription from a preincubated mouse rRNA gene promoter in a mouse S-100 cell extract can exhibit strikingly rapid kinetics, with an initial burst of synthesis in the first few minutes followed by a virtual cessation of transcription (Fig. 1). This rapid diminution in transcriptional capacity does not result from inactivation of the NTPs, the template, the stable promoter-binding transcription factors (factor D [SL1] and UBF), or the core RNA pol I elongation activity. Rather, it reflects an exhaustion of factor C* (Fig. 1 and 3), the component of the RNA polymerase I holoenzyme that is necessary for specific transcriptional initiation on a rRNA gene promoter. The ability to reactivate transcription in a transcription-exhausted extract by adding only factor C* (Fig. 1B) suggests that factor C* is the only component affected.

The rapid inactivation of factor C* upon initiation of rRNA synthesis is due to the process of transcription *per se*, since C* activity is stable during incubations in which rDNA transcription is not occurring, even when all four NTPs are present (Fig.

3, 4B, and 5A). Experiments in which transcription was blocked at various positions by using 3'dNTPs further demonstrate that C* activity is retained through transcription complex binding, initiation, and the first 33 nt of elongation but is lost before elongation has proceeded to nucleotide 54. The data in Fig. 5 indicate that C* is inactivated between 37 and 40 nt of elongation.

This transcription-dependent loss of C* activity evidently occurs independently of the sequence of the transcript being synthesized. The transcript length at which C* activity is lost, determined to be between residues 37 and 40 with the S/v template (Fig. 5), is supported by analysis with the S template, which localizes the position of C* inactivation to between residues 16 and 54 (Fig. 4). Analogous transcription-dependent loss of C* activity has been seen with all templates examined, including a mouse 3' Δ +2 deletion mutant (24) in which the entire transcript beyond the initial ApC dinucleotide is vector sequence (data not shown). The loss of C* activity is, however, specific for transcription from the rDNA promoter, since C* activity is not diminished in a reaction in which the 5S RNA gene is being transcribed (data not shown).

When a rDNA transcription reaction is not preincubated, synthesis takes place over an extended period, gradually diminishing between 30 and 60 min (Fig. 1A; data not shown). Yet, despite the marked difference in the kinetics of reactions that were and were not preincubated, similar amounts of total synthesis are obtained in these two reactions. This indicates that the rDNA preinitiation complex undergoes a relatively slow activation step before synthesis can initiate. When this activation occurs in a preincubation before NTPs are added, transcription then takes place in a rapid burst, but otherwise the synthesis period is spread out, reflecting a gradual maturation of the template complex. The same amount of total transcription in the reactions that were preincubated and not preincubated is also consistent with C* activity being used stoichiometrically in the transcription process.

A number of deductions follow from our observation that the factor C* of a transcription complex is inactivated at $\sim$ 40 nt of elongation.

(i) If C* can recycle when elongation is halted before 34 nt, a reaction should be able to produce more short (e.g., 20-, 28-, and 33-nt) than long (e.g., 54-nt) transcripts. This is observed (Fig. 4 and 6).

(ii) A reaction producing only short (e.g., 20-, 28-, and 33-nt) RNAs, in which the C* is recycling, might be expected to continue synthesis for a longer period than a reaction in which C* activity becomes lost as a result of transcribing longer (e.g., 54-nt) RNAs. This is also verified (Fig. 6A and B).

(iii) The ability of C* to recycle when synthesizing 20-, 28-, and 33-nt transcripts, yet not to transfer to a subsequently added template complex (see Results), implies that the short transcripts halted by 3'dUTP must be released from the template to allow the additional rounds of synthesis. This is also observed, because when the transcription complex is pelleted (Fig. 4 and reference 14) the 20-, 28-, and 33-nt transcripts, like longer transcripts, are released virtually quantitatively to the supernatant fraction (see Results and reference 4a).

(iv) If there is an excess of factor C* in a reaction, not all the C* would be productively bound during the preincubation, so not all could be utilized immediately upon addition of NTPs. Thus, synthesis in a reaction in which C* is in excess could extend for a substantially longer period than when the C* is limiting. The data in Fig. 6C verify such extended kinetics of synthesis in a reaction whose preincubation was supplemented with additional C*. The same protracted kinetics can be seen

whether elongation is halted at 54 nt with 3'dUTP or continues the length of the template to form a runoff transcript (data not shown). Depending on the relative amounts of C*, polymerase, and D/UBF/template complex, addition of C* can extend the kinetics of synthesis, increase the amount of synthesis obtained in the initial burst, or both (data not shown). It should be noted that not all extracts have limiting amounts of C*. Indeed, S-100 extracts prepared from actinomycin D-treated Ehrlich ascites cells (61) and from P1798 cells (37) evidently have an excess of C*. As expected, reactions involving such extracts also show protracted kinetics of synthesis, analogous to Fig. 6C, even following a preincubation (reference 37 and data not shown). Thus, the stoichiometry of the various transcription components in an extract or reconstituted reaction can prevent the transcription-dependent loss of C* activity from being apparent.

Factor C* and its relation to other polymerase I transcription components. Throughout this report, C* is called a factor because it can be stripped off of RNA polymerase I by a column resin (Fig. 2). However, C* is not an independent transcription factor, like factors D (SL1) and UBF, since under physiological conditions active C* appears quantitatively bound to RNA polymerase I; there is no evidence of free factor C* in any cell extract we have examined. C* activity remains associated with RNA polymerase I as factor C through gradient elution from seven different chromatographic matrices and through precipitation by antibodies directed against the largest subunit of RNA polymerase I, even at 1 M salt concentration (61). C* should thus be considered a subunit of the active RNA polymerase I holoenzyme, factor C.

C* may be the same activity as TFIC (identified in the Thompson laboratory), which is lacking in extracts prepared from the mutant cell line P1798 following transcriptional down-regulation with glucocorticoids. Like C*, TFIC is strongly associated with RNA polymerase I and its activity is very rapidly lost upon addition of NTPs to a transcription complex in which TFIC is limiting (10, 17, 38). However, it remains to be demonstrated whether and where TFIC becomes exhausted during the rDNA transcription process and whether TFIC can stimulate transcription in extracts of stationary-phase or cycloheximide-treated cells. Notably, and in contrast to C*, there appears to be a large amount of the TFIC activity; it has been attributed to 55-, 50-, and 42-kDa polypeptides (38) that are considerably more abundant than the RNA polymerase I itself (39) and are reported to be ~10% transcriptionally active (37). Relative to the small amount of C* activity in our S-100 extracts of L1210, Ehrlich ascites, and HeLa cells, this would imply that there are 2 to 3 orders of magnitude more TFIC activity in the S-100 extracts of P1798 cells, a rather surprising conclusion if factor C* and TFIC are indeed the same component.

It might also seem likely that factor C* is the same component as factor TIF-IA identified in the Grummt laboratory (6, 54, 55), because both restore in vitro rDNA transcription to inactive extracts prepared from stationary-phase or protein synthesis-inhibited cells and both bind to RNA polymerase I. However, the purification characteristics of these two components appear rather distinct, with C* eluting differently from polymerase I on Mono Q chromatography (Fig. 2) but not on heparin chromatography (61) and TIF-IA eluting differently from polymerase I on heparin chromatography but not on Mono Q chromatography (54). Also, TIF-IA has recently been reported to recycle and not be consumed in the transcription process (55). Thus TIF-IA might not be C* but might be an activity present in the extracts prepared in the Grummt laboratory that can reactivate C* once it has been inactivated

by transcription. However, the experiment to demonstrate TIF-IA recycling appears to have been set up with excess TIF-IA activity (55), which could make it appear to recycle even if it were being consumed by the transcription process (as in Fig. 6C).

Finally, in apparent analogy to the loss of factor C activity in growth-arrested mammalian cells, encysting cells of the soil amoeba *Acanthamoeba castellanii* also demonstrate down-regulation of rRNA gene transcription as a result of loss of specific initiation competence by the RNA polymerase I (1, 48). No specific initiation factor has been separated from the *A. castellanii* RNA polymerase I catalytic activity, but the *A. castellanii* RNA polymerase I (which has twice as many copurifying polypeptides as does the mammalian enzyme) could bind its C* equivalent more tightly. In addition, the active *A. castellanii* polymerase I is able to catalyze multiple rounds of transcription in vitro, so if it undergoes a process analogous to C* inactivation, associated polypeptides must be able to reactivate it.

Transcription-dependent inactivation of C* also occurs in vivo. Our data indicate that the transcription-dependent inactivation of C* observed in vitro is representative of the in vivo rDNA transcription process. For two decades, it has been known that rRNA gene transcription is selectively down-regulated when cellular protein synthesis is shut off, as by cycloheximide or pactomycin treatment (12, 18, 32, 59, 65). This has led to the conclusion that an inherently short-lived polypeptide is required for rRNA gene transcription (4, 11, 12, 15, 18, 29, 32, 36, 44–46, 59, 65). We and others have since shown that transcriptional down-regulation is mimicked in extracts of protein synthesis-inhibited cells (see, e.g., references 16, 20, and 42) and that this is due to a lack of factor C activity (16, 54, 61), specifically the C* component of factor C (Fig. 2), suggesting that C* is the hypothesized short-lived protein.

The data reported in this paper show that C* activity is not inherently unstable in vivo in the absence of protein synthesis, as might have been thought. Indeed, cellular C* activity is depleted only when rRNA gene transcription is taking place while the protein synthesis is inhibited (Fig. 7). The cellular C* remains active in cells in which rRNA gene transcription is not taking place, even in the absence of protein synthesis. These results indicate that in vivo, as in vitro, C* activity is consumed during the process of rRNA gene transcription. In untreated growing cells, factor C* would have to be replenished to compensate for its inactivation in the rDNA transcription process. The replenishment of C* activity requiring protein synthesis (Fig. 7) suggests either that a short-lived protein (such as a phosphatase or kinase) is necessary to reactivate C* or that the C* polypeptides themselves have to be resynthesized. It should also be noted that the similarity of the transcription-dependent loss of C* activity that occurs in vivo and in vitro strongly indicates that the effects observed with extracts reflect the in vivo situation and argues against the in vitro inactivation of C* being artifactual.

Mode of C* inactivation. From our in vitro data, the transcription-dependent C* inactivation cannot be simply a dissociation of active C* from the polymerase, because then the released C* should bind other polymerase molecules of the extract and support further transcription, just like added isolated C* does (Fig. 1B). Our data also indicate that inactivation of C* is not due to its sequestration on the template complex (as can occur with RNA polymerase II in vitro [51]) or to its sequestration on the released transcript (Fig. 3 and associated experiments). The finding of C* inactivation occurring independently of the transcript sequence also supports

this latter conclusion. In addition, a battery of protease inhibitors did not affect C* inactivation (data not shown), consistent with the process not involving proteolysis. Thus, it seems likely that the transcription-dependent inactivation of factor C* involves a posttranslational modification. Unfortunately, the very low abundance of active C* in our extracts has prevented our definitive identification of its constituent polypeptides, despite a substantial effort (4b). If factor C* is the same as TFIC identified in the Thompson laboratory (38, 39), inactivation should be a posttranslational modification, since the 55-, 50-, and 42-kDa polypeptides identified as TFIC appear the same when prepared from cells down-regulated for rDNA transcription (39). Also, in *A. castellanii* a posttranslational modification would be most consistent with the presumed difference between initiation-competent and incompetent forms of RNA polymerase I, because the 39-kDa RNA polymerase I subunit shows a slightly different apparent size in encysting cells (26). For RNA polymerase II, initiation and elongation forms also differ by a posttranslational modification (see below).

Relation to other polymerases. The mouse factors C and C* appear reminiscent of *Escherichia coli* RNA polymerase holoenzyme and its σ subunit, respectively (5, 22; see also reference 10). In both cases, a specific initiation-competent holoenzyme can be chromatographically separated into a specific initiation competence-conferring component lacking polymerase activity and a core polymerase that catalyzes elongation but lacks specific initiation competence. Also, in both cases the specific initiation competence is lost from the holoenzyme relatively early in the elongation process.

The transition of an initiation-competent RNA polymerase into an elongation form early in the transcription process may be a fairly general theme, occurring not only with prokaryotic RNA polymerase and RNA polymerase I but also with RNA polymerase II (28, 33). There are, however, differences in the nature of the changes and the positions where they occur. With prokaryotic RNA polymerase, the transition occurs when the σ subunit dissociates from the core enzyme at ~10 nt of elongation (22). The vertebrate RNA polymerase I transition also involves the initiation specificity subunit of the holoenzyme, but the transition involves inactivation of factor C* and occurs when elongation has proceeded ~37 nt. With RNA polymerase II, the change is phosphorylation of the C-terminal heptad repeat of the largest polymerase subunit, and it evidently occurs before elongation has begun (28, 33). Whereas the dissociated σ subunit is ready to recycle to another polymerase (62) and the phosphorylated RNA polymerase II also presumably recycles following a phosphatase action, reactivation of inactivated factor C* remains to be demonstrated. In all these cases, the change in the holoenzyme could enable the polymerase to be both highly specific for the promoter at initiation and able to move away from the promoter down the template during elongation.

Alterations in factor C are a common mode of rDNA transcriptional regulation. It may be that with RNA polymerase I the alteration between initiation-competent and initiation-incompetent forms has also adopted a specific transcriptional regulatory role. Such a function would not be possible with RNA polymerase II or *E. coli* RNA polymerase, which are responsible for transcribing thousands of different genes. As noted above, a loss of active C* is observed or inferred in nearly all studied cases of in vivo rDNA transcriptional down-regulation, including protein synthesis inhibition (induced by various drug treatments or by amino acid starvation), serum starvation, hormonally induced down-regulation, and poliovirus infection. A complementary C* increase is

observed or inferred upon reversal of these conditions. Thus, studies of factor C* and its inactivation should help provide an understanding not only of the process of rDNA transcription but also of many of the described cases of rDNA transcriptional regulation.

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REFERENCES

1. Bateman, E., and M. R. Paule. 1986. Regulation of eukaryotic ribosomal RNA transcription by RNA polymerase modification. *Cell* 47:445-450.
2. Bell, S. P., H.-M. Jantzen, and R. Tjian. 1990. Assembly of alternative multiprotein complexes directs rRNA promoter selectivity. *Genes Dev.* 4:943-954.
3. Bell, S. P., R. M. Learned, H.-M. Jantzen, and R. Tjian. 1988. Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science* 241:1192-1197.
4. Benecke, B. J., A. Ferencz, and K. H. Seifart. 1973. Resistance of hepatic RNA polymerases to compounds effecting RNA and protein synthesis in vivo. *FEBS Lett.* 31:53-58.
- 4a. Brun, R. Unpublished observations.
- 4b. Brun, R., and E. Nikolov. Unpublished observations.
5. Burgess, R. R., A. A. Travers, J. J. Dunn, and E. K. F. Bautz. 1969. Factor stimulating transcription by RNA polymerase. *Nature (London)* 221:43-46.
6. Buttgerit, D., G. Pflugfelder, and I. Grummt. 1985. Growth-dependent regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA.) *Nucleic Acids Res.* 13:8165-8180.
7. Cavanaugh, 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.
8. Cavanaugh, A. H., and E. A. Thompson, Jr. 1983. Hormonal regulation of transcription of rDNA: inhibition of transcription during glucocorticoid-mediated inhibition of proliferation of lymphosarcoma P1798 cells in culture. *J. Biol. Chem.* 258:9768-9773.
9. Cavanaugh, A., 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.
10. Cavanaugh, A. H., and E. A. Thompson, Jr. 1986. Hormonal regulation of rDNA: initiation of transcription by RNA polymerase I *in vitro*. *J. Biol. Chem.* 261:12738-12744.
11. Cereghini, S., and M. T. Franze-Fernandez. 1974. Ehrlich ascites cells DNA-dependent RNA polymerases: effect of amino acids and protein synthesis inhibition. *FEBS Lett.* 41:161-165.
12. Chesterson, C. J., B. E. H. Coupar, P. H. W. Butterworth, J. Buss, and M. H. Green. 1975. Studies on the control of ribosomal RNA synthesis in HeLa cells. *Eur. J. Biochem.* 57:79-83.
13. Comai, L., N. Tanese, and R. Tjian. 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell* 68:965-976.
- 13a. Culotta, V., and B. Sollner-Webb. Unpublished observations.
14. Culotta, V. C., R. J. 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.* 5:1582-1590.
15. Franze-Fernandez, M. T., and A. V. Fontanive-Sanguesa. 1975. Control of rRNA synthesis: effect of protein synthesis inhibition. *FEBS Lett.* 54:26-29.
16. Gokal, P. K., A. H. Cavanaugh, and E. A. Thompson, Jr. 1986. The

- effects of cycloheximide upon transcription of rRNA, 5S RNA, and tRNA genes. *J. Biol. Chem.* **261**:2536–2541.
17. Gokal, P. K., P. B. Mahajan, and E. A. Thompson, Jr. 1990. Hormonal regulation of transcription of rDNA: formation of initiated complexes by RNA polymerase I *in vitro*. *J. Biol. Chem.* **265**:16234–16243.
 18. Gross, K. J., and A. O. Pogo. 1976. Control of ribonucleic acid synthesis in eukaryotes. 3. The effect of cycloheximide and edeine on RNA synthesis in yeast. *Biochemistry* **15**:2082–2086.
 19. Grummt, I. 1978. *In vitro* synthesis of Pre-rRNA in isolated nucleoli. *Cell Nucleus* **5**:373–415.
 20. 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.
 21. Grummt, I., V. A. Smith, and F. Grummt. 1976. Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. *Cell* **7**:439–445.
 22. Hansen, U. M., and W. R. McClure. 1980. Role of the sigma subunit of *Escherichia coli* RNA polymerase in initiation: release of sigma from ternary complexes. *J. Biol. Chem.* **255**:9564–9570.
 23. Henderson, S. L., K. Ryan, and B. Sollner-Webb. 1989. The promoter-proximal rDNA terminator augments initiation by preventing disruption of the stable transcription complex caused by polymerase read-in. *Genes Dev.* **3**:212–223.
 24. Henderson, S. L., and B. Sollner-Webb. 1990. The mouse ribosomal DNA promoter has more stringent requirements *in vivo* than *in vitro*. *Mol. Cell. Biol.* **10**:4970–4973.
 25. Hoeffler, W. K., and R. G. Roeder. 1985. Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. *Cell* **41**:955–963.
 26. Iida, C. T., and M. R. Paule. 1992. Purification of components required for accurate transcription of ribosomal RNA from *Acanthamoeba castellanii*. *Nucleic Acids Res.* **20**:3211–3221.
 27. Jantzen, H.-M., A. Admon, S. P. Bell, and R. Tjian. 1990. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature (London)* **344**:830–836.
 28. Kang, M. E., and M. E. Dahmus. 1993. RNA polymerases IIA and IIO have distinct roles during transcription from the TATA-less murine dihydrofolate reductase promoter. *J. Biol. Chem.* **268**:25033–25040.
 29. Karagoyozov, L. K., B. B. Stoyanova, and A. A. Hadjiolov. 1980. Effect of cycloheximide on the *in vivo* and *in vitro* synthesis of ribosomal RNA in rat liver. *Biochim. Biophys. Acta* **607**:295–303.
 30. Kuhn, A., and I. Grummt. 1992. Dual role of the nucleolar transcription factor UBF: trans-activator and antirepressor. *Proc. Natl. Acad. Sci. USA* **89**:7340–7344.
 31. Kuhn, A., V. Stefanovsky, and I. Grummt. 1993. The nucleolar transcription activator UBF relieves Ku-antigen mediated repression of mouse ribosomal gene transcription. *Nucleic Acids Res.* **21**:2057–2063.
 32. Lampert, A., and P. Feigelson. 1974. A short lived polypeptide component of one of two discrete functional pools of hepatic nuclear α -amanitin resistant RNA polymerases. *Biochem. Biophys. Res. Commun.* **59**:1030–1038.
 33. Laybourn, P. J., and M. E. Dahmus. 1990. Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. *J. Biol. Chem.* **265**:13165–13173.
 34. 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.
 35. Learned, R. M., T. K. Learned, M. M. Haltiner, and R. T. Tjian. 1986. Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. *Cell* **45**:847–857.
 36. Lindell, T. J., A. F. O'Malley, and B. Puglisi. 1978. Inhibition of nucleoplasmic transcription and the translation of rapidly labeled nuclear proteins by low concentrations of actinomycin D *in vivo*. Proposed role of messenger RNA in ribosomal RNA transcription. *Biochemistry* **17**:1154–1160.
 37. Mahajan, P. B., P. K. Gokal, and E. A. Thompson, Jr. 1990. Hormonal regulation of transcription of rDNA: the role of TFIC in formation of initiation complexes. *J. Biol. Chem.* **265**:16244–16247.
 38. Mahajan, P. B., and E. A. Thompson, Jr. 1990. Hormonal regulation of transcription of rDNA: purification and characterization of the hormone-regulated transcription factor IC. *J. Biol. Chem.* **265**:16225–16233.
 39. Mahajan, P. B., and E. A. Thompson, Jr. 1992. Co-purification of RNA polymerase I and the glucocorticoid-regulated transcription factor IC. *Protein Exp. Purif.* **3**:410–416.
 40. McStay, B., C. H. Hu, C. S. Pikaard, and R. H. Reeder. 1991. xUBF and Rib1 are both required for formation of a stable polymerase I promoter complex in *X. laevis*. *EMBO J.* **10**:2297–2303.
 41. 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.
 42. Miller, K. G., and B. Sollner-Webb. 1982. Transcription of mouse ribosomal RNA genes. *Cell Nucleus* **12**:69–100.
 43. Mishima, Y., I. Financsek, R. Kominami, and M. Muramatsu. 1982. Fractionation and reconstitution of factors required for accurate transcription of mammalian rRNA genes: identification of a species-dependent initiation factor. *Nucleic Acids Res.* **10**:6659–6670.
 44. Mishima, Y., T. Matsui, and M. Muramatsu. 1979. The mechanism of decrease in nucleolar RNA synthesis by protein synthesis inhibition. *J. Biochem.* **85**:807–818.
 45. Muramatsu, M., M. Matsui, T. Onishi, and Y. Mishima. 1979. Nucleolar RNA polymerase and transcription of nucleolar chromatin. *Cell Nucleus* **7**:123–161.
 46. Muramatsu, M., N. Shimada, and T. Higashinakagawa. 1970. Effect of cycloheximide on the nucleolar RNA synthesis in rat liver. *J. Mol. Biol.* **53**:91–106.
 47. Pape, L. K., J. J. Windle, and B. Sollner-Webb. 1990. Half helical turn spacing changes convert a frog to a mouse rDNA promoter: a distant upstream domain determines the helix face of the initiation site. *Genes Dev.* **4**:52–62.
 48. Paule, M. R., C. T. Iida, P. J. Perna, G. H. Harris, D. A. Knoll, and J. M. D'Allesio. 1984. *In vitro* evidence that eukaryotic ribosomal RNA transcription is regulated by modification of RNA polymerase I. *Nucleic Acids Res.* **12**:8161–8180.
 49. Perry, R. P., and D. E. Kelley. 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. *J. Cell. Physiol.* **76**:127–140.
 50. Rubenstein, S. J., and A. Dasgupta. 1989. Inhibition of rRNA synthesis by poliovirus: specific inactivation of transcription factors. *J. Virol.* **63**:4689–4696.
 51. Sawadogo, M., and R. G. Roeder. 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43**:165–175.
 52. Schnapp, A., J. Clos, W. Hadelt, R. Schreck, A. Cvekl, and I. Grummt. 1990. Isolation and functional characterization of TIF-IB, a factor that confers promoter specificity to mouse RNA polymerase I. *Nucleic Acids Res.* **81**:1385–1393.
 53. Schnapp, A., and I. Grummt. 1991. Transcription complex formation at the mouse rDNA promoter involves the stepwise association of four transcription factors and RNA polymerase I. *J. Biol. Chem.* **266**:24588–24595.
 54. Schnapp, A., C. Pfeleiderer, H. Rosenbauer, and I. Grummt. 1990. A growth-dependent transcription initiation factor (TIF-IA) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. *EMBO J.* **9**:2857–2863.
 55. Schnapp, A., G. Schnapp, B. Erny, and I. Grummt. 1993. Function of the growth-regulated transcription initiation factor TIF-IA in initiation complex formation at the murine ribosomal gene promoter. *Mol. Cell. Biol.* **13**:6723–6732.
 56. Smith, S. D., E. Oriahi, D. Lowe, H.-F. Yang-Yen, D. O'Mahony, K. Rose, K. Chen, and L. I. Rothblum. 1990. Characterization of factors that direct transcription of rat ribosomal DNA. *Mol. Cell. Biol.* **10**:3105–3116.
 57. Sollner-Webb, B., and E. B. Mougey. 1991. News from the nucleolus: rRNA gene expression. *Trends Biochem. Sci.* **16**:58–62.
 58. Sollner-Webb, B., and J. Tower. 1986. Transcription of cloned

- eukaryotic ribosomal RNA genes. *Annu. Rev. Biochem.* **55**:801–830.
59. Suzuki, N., T. Hosoya, T. Igarashi, and M. Muramatsu. 1984. Changes in template-engaged and free RNA polymerase I activities in isolated nuclei from rat ventral prostates after treatment with testosterone and cycloheximide. *J. Biochem.* **95**:1389–1398.
60. Tower, J., V. C. Culotta, and B. Sollner-Webb. 1986. Factors and nucleotide sequences that direct ribosomal DNA transcription and their relationship to the stable transcription complex. *Mol. Cell. Biol.* **6**:3451–3462.
61. Tower, J., and B. Sollner-Webb. 1987. Transcription of mouse rDNA is regulated by an activated subform of RNA polymerase I. *Cell* **50**:873–883.
62. Travers, A. A., and R. R. Burgess. 1969. Cyclic re-use of the RNA polymerase sigma factor. *Nature (London)* **222**:537–540.
63. Vallett, S. M., M. Brudnak, M. Pellegrini, and H. W. Weber. 1993. In vivo regulation of rRNA transcription occurs rapidly in nondividing and dividing *Drosophila* cells in response to a phorbol ester and serum. *Mol. Cell. Biol.* **13**:928–933.
64. Xie, W., D. J. O'Mahony, S. D. Smith, D. Lowe, and L. I. Rothblum. 1992. Analysis of the rat ribosomal RNA promoter: characterization of linker-scanning mutants and of the binding of UBF. *Nucleic Acids Res.* **20**:1587–1592.
65. Yu, F. L., and P. Feigelson. 1972. The rapid turnover of RNA polymerase of rat liver nucleolus, and of its messenger RNA. *Proc. Natl. Acad. Sci. USA* **69**:2833–2837.