MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3418–3427
0270-7306/86/103418-10$02.00/0

Vol. 6, No. 10

Formation of the Transcription Initiation Complex on Mammalian rDNA

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Received 4 February 1986/Accepted 16 June 1986

Steps for the formation of transcription initiation complex on the human rRNA gene (rDNA) in vitro were analyzed with partially purified transcription factors and RNA polymerase I. The reaction requires at least two factors besides RNA polymerase I for maximal efficiency. Preincubation and short-pulse analyses of the accurate transcripts revealed the following steps. First, the species-dependent factor, designated TFID, bound to the rDNA template, forming a preinitiation complex (PIC-1) which was resistant to a moderate concentration (0.015 to 0.02%) of Sarkosyl. Other factors, designated TFIA and RNA polymerase I, were then added to convert it to the final preinitiation complex PIC-3. This complex incorporated the first two nucleoside triphosphates of the starting site to complete the initiation complex (IC), which was resistant to a high concentration (0.2%) of Sarkosyl. Binding of TFID was rate limiting in the overall initiation reaction in vitro. Together with the kinetics of incorporation, the results are interpreted to mean that TFID, one bound, remains complexed with rDNA together with TFIA as the PIC-2 for many rounds of transcription by RNA polymerase I. Thus, the formation of PIC-2 may be a prerequisite for the stable opening of rDNA for transcription in vivo.

Transcription in eucaryotic cells generally requires multiple factors besides the RNA polymerase for each class of the gene. For RNA polymerase III, which transcribes tRNA, 5S rRNA, and virus-associated RNA, at least two or three factors have been shown to be necessary for accurate and efficient transcription initiation depending upon the gene to be transcribed (30, 32), and the mechanisms of their interaction with template DNA are considerably clarified (2, 9, 31).

Similar attempts for RNA polymerase II, which transcribes protein-coding genes and most of the small nuclear RNAs, are also under way (6, 7, 11, 19, 28, 37), and a few factors have been found to be required in either a gene-specific or a nonspecific manner. Their binding to specific region of template DNA has also been shown (8, 26, 27, 29).

As for the RNA polymerase I system which transcribes the rRNA gene (rDNA) almost exclusively, some progress has been made in the analysis of transcription processes since the development of in vitro transcription systems by workers in several laboratories including ours (12, 15, 18, 21, 23, 25). We fractionated a mouse cell extract (S-100) by phosphocellulose chromatography. Then we demonstrated that, as for systems of RNA polymerase II and III, multiple factors are required for a correct and efficient transcription of rDNA and that the in vitro system could be reconstituted from the fractionated components, the fraction D that eluted at a high salt concentration being indispensable for accurate initiation (22, 24). We also demonstrated that the factor contained in this fraction, designated TFID in this paper, was responsible for the species dependency of the RNA polymerase I transcription machinery described by Kohorn and Rae (15) and Grummt et al. (12, 13). More recently, Wandelt and Grummt (38) and Cizewski and Sollner-Webb (5), using the unfractionated extract, presented data suggesting the formation of a stable transcription complex that may survive many rounds of reinitiation on mouse rDNA. Miesfeld and Arnheim (22), in the meantime, showed that the factor mentioned above is responsible for the complex formation which requires the promoter region of rDNA. The key role played by TFID now seems apparent, but the precise kinetics of its interaction with template DNA and the interactions of other possible factors and polymerase I with the template have not been established so far.

In this paper, we describe a detailed analysis of the formation of the transcription initiation complex of a human rRNA gene in vitro. We found that TFID binds first with the template, which is followed by the binding of the factor(s), designated TFIA here, in another fraction and RNA polymerase I. We suggest, in addition, that TFID remains bound to the template while RNA polymerase I transcribes many rounds on the template. We further dissected the initiation reaction into two different steps by the resistance to a detergent, Sarkosyl (CIBA-GEIGY Corp.). The preinitiation complex, before addition of initiating nucleotides, had a distinct sensitivity to this detergent compared with that of the initiation complex with the first two nucleotides at the initiation site.

MATERIALS AND METHODS

Fractionation of cell extracts. HeLa and FM3A cells were grown in suspension culture. A cell extract (S-100) was prepared as described by Weil et al. (39) with a slight modification (35). The supernatant, after centrifugation at 10,000 × *g*, was precipitated by addition of solid ammonium sulfate to 60% saturation to remove low-molecular-weight materials, including most of the nucleoside triphosphates. The pellet was suspended in a small volume of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]–KOH [pH 7.9], 20% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol) and desalted on a Sephadex G-25 column equilibrated with buffer A containing 0.1 M KCl and dialyzed against the same buffer for 3 h. The dialyzed extract was loaded onto a phosphocellulose (P-11) column equilibrated with buffer A containing 0.1 M KCl. Chromatography was performed as described previously (24), except that fractions B and C were eluted together at 0.6 M KCl. This fraction was dialyzed and applied onto a DEAE-cellulose (DE-52) column equilibrated with buffer A containng 0.1 M KCl and 5 mM

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TABLE 1. Summary of purification of the fractions

Fraction	Total vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Approx. puification factor
S-100	30	16	480	1
A	43	9.6	413	1
B + C	21	1.9	40	7–8
D	21	0.20	4.2	100
A′	37	1.1	41	8
P	7.6	0.83	6.3	40

$MgCl_2$. After the column was washed with the same buffer, a fraction (P) containing most of the RNA polymerase I activity was step-eluted at 0.3 M KCl in the same buffer. Fraction A was also further chromatographed in a DEAE-cellulose column to obtain fraction A′. It was eluted at 0.3 M KCl after the column was washed with 0.1 M KCl in buffer A. This procedure not only purified the fraction considerably with respect to TFIA but also efficiently removed the remaining nucleoside triphosphates. Activity in fraction A′ also appeared in the next 0.6 M KCl eluate considerably but was not used in this study. All fractions were dialyzed against 0.1 M KCl in buffer A and used for the reconstitution system. Typical values obtained from a series of purification with 15 g of HeLa cells are given in Table 1. Protein concentrations were determined as described previously (3).

In vitro RNA synthesis. In vitro transcription was carried out at 30°C for 60 min in a 25 μl reaction mixture containing 10 mM HEPES-KOH (pH 7.9), 10% glycerol, 80 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, and 100 μg of α-amanitin per ml. Nucleotide concentrations were 500 μM each for ATP, GTP, and CTP and 25 μM for UTP containing 5 μCi of [α-^{32}P]UTP (400 Ci/mmol; Amersham). The reaction conditions are given in the figure legends; otherwise, the reactions were performed under standard conditions in which 2 μl of fraction A′, 4 μl of fraction P, 4 μl of fraction D, and 20 μg of template DNA per ml were included. These volumes do not reflect the amount contained in the original cell extract but rather represent optimal amounts required for accurate and efficient transcription initiation in the reconstituted system. In fact, the amount of fraction A′ was about 10 times lower in S-100 than was fraction P or fraction D. Reactions were terminated by the addition of 200 μl of stop buffer (7 M urea, 0.1% sodium dodecyl sulfate, 10 mM Tris-hydrochloride [pH 7.9], 10 mM EDTA, and 100 μg of tRNA per ml), and the mixtures were extracted with phenol-chloroform-isoamyl alcohol (20:20:1) and then with chloroform. After ethanol precipitation, nucleic acid pellets were glyoxalated (20) in a small volume (20 μl) and directly electrophoresed on a 2% agarose gel in 10 mM sodium phosphate (pH 6.9)–0.5 mM EDTA. The amount of specific transcript synthesized under standard conditions was about 2×10^{-3} to 3×10^{-3} pmol in 60 min at 30°C, which corresponds to about 1 to 1.5% of the template molecules, if it is assumed that only one round of transcription occurred. Human rDNA template was prepared by cutting a human rDNA clone (pBE2Δ) which was derived from pBE2 (41) with *Sal*I or *Pvu*II. Mouse rDNA template described previously (25) was cut with *Pvu*II. Four ribonucleoside triphosphates were purchased from Boehringer Mannheim Biochemicals.

Preincubation and short-pulse analysis. The preincubation and short-pulse protocol was described previously in principle (11). In most experiments, the preincubation was performed for 60 min. Pulse-labeling was allowed to continue for 2 min with 250 μM each ATP, GTP, and CTP and 10 μM UTP containing 5 μCi of [α-^{32}P]UTP (400 Ci/mmol) and was followed by a chase for 3 min after the addition of 1 mM unlabeled UTP. For binding order analysis, two-step preincubations, one each for 10 min and 30 min, were also used. For sequestration-type experiments, two templates were prepared. The plasmid pBE2Δ was cut either with *Sal*I or *Pvu*II to give a template producing a 696- or 2,111-nucleotide transcript each. The standard conditions were maintained throughout the preincubations and the transcription reaction. The concentration of Sarkosyl indicated at each step was also maintained by the addition of concentrated Sarkosyl.

RESULTS

Fractionation and dose-response characteristics of transcription components from HeLa cell extract. HeLa cell extract S-100, prepared as described in Materials and Methods, was fractionated by the method described previously (24), except that fractions B and C were eluted together with 0.6 M KCl and further purified with respect to RNA polymerase I on a column of DEAE-cellulose, yielding a fraction designated P (Fig. 1A). Fraction A was also further purified on a DEAE-cellulose column to produce fraction A′.

All three fractions, A′, P, and D, are required for the accurate and efficient transcription initiation of human rDNA by the HeLa RNA polymerase I system (Fig. 1B, lane 7). No accurate transcription occurred without fraction D (lane 4), as was found previously (22, 24). Omission of either fraction A′, or fraction P did not give a correct transcript (lanes 6 and 5). Fraction P alone gave rise to mostly end-to-end transcription products.

Although this in vitro transcription system was not completely dependent upon fraction P (partially purified RNA polymerase I), a dose-response experiment showed that up to a 30-fold augmentation of transcription may be obtained when fraction P is added to a certain level (Fig. 2B). Fraction A′ stimulated an accurate transcription more than 25 times, even at a low level (Fig. 2A). For fraction D, the transcription increased almost linearly up to the highest amount tested (Fig. 3C). The reaction was also dependent on the concentration of rDNA template, as might be expected, but too high a DNA concentration became inhibitory to correct transcription and rather increased nonspecific initiations (Fig. 2D). The results suggest that although all these fractions are required for accurate and efficient initiation of transcription on rDNA, the factor(s) in fraction A′ is present in excess in HeLa cell extract, taking into account the fractionation data shown in Materials and Methods. These dose-response curves also suggest that cross-contamination between fractions is negligible for the purpose of further analyses.

Formation of transcription initiation complex in vitro requires a certain time. As a first step in the analysis of the formation of the initiation complex on rDNA, we examined the time course of transcription of rDNA template with or without preincubation by using fractionated components. Figure 3A clearly shows that there was a definite time lag before transcription starts in full when all the fractions are incubated together with substrates. This lag was abolished when the mixture was preincubated for 60 min before the addition of substrates. These data strongly suggest that a certain time is required for the formation of the transcription initiation complex in vitro. To assess the length of time required for formation of the initiation complex under stan-

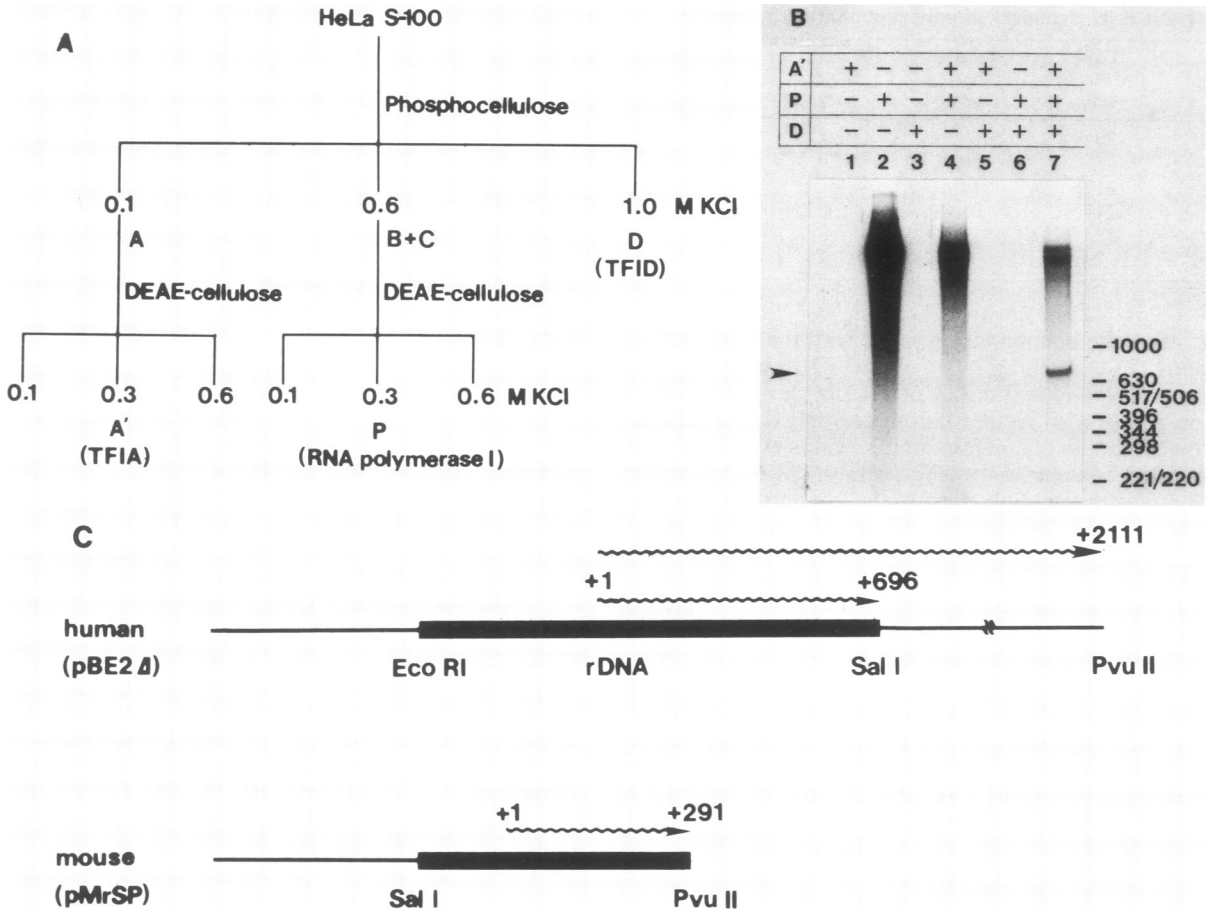

FIG. 1. Scheme of fractionation of HeLa cell extract, transcription with reconstituted fractions, and templates used for in vitro transcription. (A) A cell extract (S-100) prepared from HeLa cells was chromatographed on a phosphocellulose column and then on a DEAE-cellulose column, as described in Materials and Methods. Three fractions were step eluted from the phosphocellulose column at 0.1 M KCl (flow-through [A]), 0.6 M KCl (B + C) and 1.0 M KCl (D). The flow-through (A) and the eluate at 0.6 M KCl (B + C) were further chromatographed on a DEAE-cellulose column. The eluates with 0.3 M KCl, after being washed with 0.1 M KCl, were termed A′ and P, respectively. Fraction P contained most of RNA polymerase I activity. (B) Autoradiograph of the gel analysis of RNA products transcribed in the reconstituted systems. The combinations of the fractions (A′, P, and D) are indicated by + or − at the top. Lanes: 1, 2 μl of A′; 2, 4 μl of P; 3, 4 μl of D; 4, 2 μl of A′ + 4 μl of P; 5, 2 μl of A′ + 4 μl of D; 6, 4 μl of P + 4 μl of D; 7, 2 μl of A′ + 4 μl of P + 4 μl of D. The reaction was carried out in the presence of 0.5 μg of rDNA template (*Sal*I-cut pBE2Δ; see panel C) in a 25-μl mixture for 60 min at 30°C. The arrowhead shows the 696-nucleotide runoff transcript that accurately initiated from the same start point as in vivo. Markers are products of ^{32}P-end-labeled *Eco*RI-*Hin*fI fragments of pBR322. The smears observed in lanes 2 and 6 were not always so strong (see, for example, Fig. 2). They were influenced mainly by fraction P, which had smear-suppressing activity, probably as a result of poly(ADP-ribose) polymerase (33). (C) Templates. Plasmid pBE2Δ is a derivative of pBE2 containing the 6.1-kilobase *Eco*RI fragment of human rDNA. It consists of the vector pBR322 (thin line) and a 1.2-kilobase fragment containing the initiation site of human rDNA (solid bar). In most experiments, the template was prepared by cutting pBE2Δ with *Sal*I. pBE2Δ cut with *Pvu*II was also used in sequestration-type experiments. Plasmid pMrSP consists of the vector pBR322 (thin line) and a 0.46-kilobase fragment containing the initiation site of mouse rDNA (solid bar). The wavy lines show the runoff transcripts.

dard conditions, we changed the preincubation time and estimated the amount of the initiation complex by a short-pulse experiment. The short-pulse protocol, which involves a short labeling of the correct transcript with [α-^{32}P]UTP followed by the completion of the RNA chain in the presence of cold UTP, can be used to analyze the amount of initiation complex with minimum perturbations by the elongation reaction (11). The results shown in Fig. 3B clearly indicate that about 40 min are required for the full formation of the initiation complex under these conditions.

TFID first binds with the rDNA. To investigate the order in which the transcription factors bind to the template rDNA, preincubation and short-pulse experiments were carried out with the omission of one or more fractions during the preincubation. For this purpose, a preliminary experiment was carried out to determine the elongation rate of initiated RNA chain by RNA polymerase I. Two templates of different lengths were preincubated with three fractions, A′, P, and D, for 30 min, and transcription was started by the addition of the four nucleoside triphosphates. The runoff products on the template truncated at position +696 appeared after about 1.5 min (Fig. 4A, lane 4), while those on the template truncated at position +2111 became clear after 3 min (Fig. 4A, lane 6). These data indicated that the elongation rate is 500 to 700 nucleotides per min. This means that a 2-min pulse followed by a 3-min chase will certainly be enough to recover all the initiated chains as a 696-nucleotide band under the conditions described. Omission of fraction D

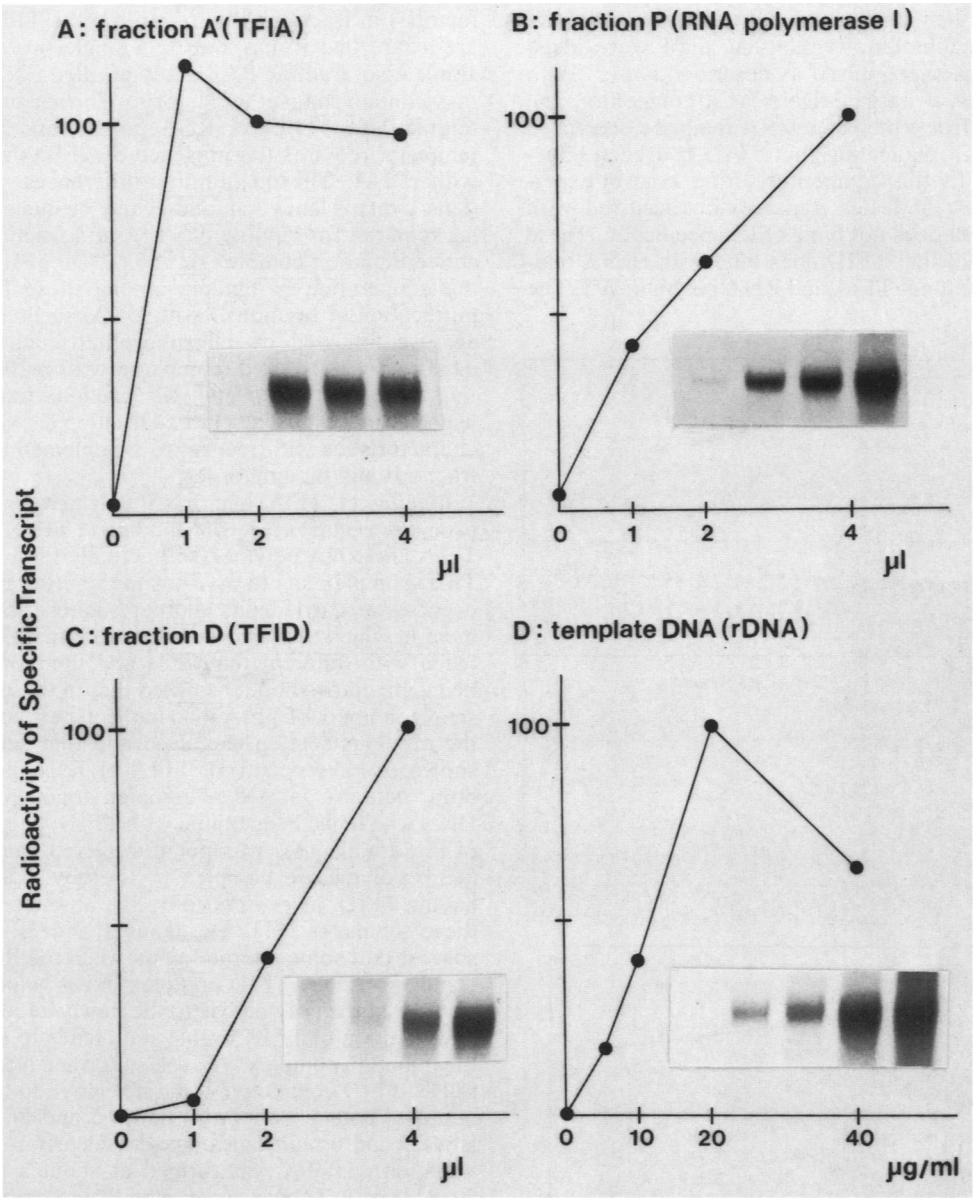

FIG. 2. Dose-response curves for transcriptional components. All reactions were based on the standard reaction conditions that contained 2 μl of A', 4 μl of P, 4 μl of D, and 20 μg of template DNA per ml in a 25-μl reaction mixture. Only one of these parameters was varied. All autoradiographs were quantitated by densitometric tracing, and the quantity of specific transcripts was plotted. All values were normalized by adjusting the amount of transcript under standard conditions to 100%. (A) Dose-response curve for fraction A'. Fraction A' (0 to 4 μl) was added to the standard reaction mixture containing all the components but A'. (B) Dose-response curve for fraction P. Fraction P (0 to 4 μl) was added to the standard reaction mixture minus P. (C) Dose-response curve for fraction D. Fraction D (0 to 4 μl) was added to the standard reaction mixture minus D. (D) Dose-response curve for template DNA. The *Sal*I-cut pBE2Δ (0 to 40 μg/ml) was added to the standard reaction mixture lacking template DNA.

during preincubation almost eliminated the transcription initiation (Fig. 4B), as might be expected from the results in Fig. 3. The very faint band seen in lane 2 may be due to the small amounts of initiation complexes formed during the pulse. On the contrary, omission of fraction A' or fraction P during preincubation inhibited the transcription only to a certain extent: about one-fourth the amount transcribed with complete preincubation was obtained (lanes 3 and 4). Preincubation with fraction D alone also provided about a 10% yield of the transcript (lane 7). The results shown in lanes 2, 5, 6, and 8 confirm that the presence of fraction D during preincubation is essential for a significant transcription to occur.

The results strongly suggest that a factor in fraction D, designated TFID, must bind to the template rDNA as the first step of rDNA activation. The possibility that the binding of TFID only takes much longer than that of other factors can be ruled out by the experiment described in the next section.

To confirm that TFID does bind to the template to make a complex, we next carried out a sequestration-type experiment in which fraction D was preincubated with a competi-

tor template and then challenged with a tester template. After the second incubation, fractions A′ and P were added and the system was short-pulsed as described above. With increasing amounts of human rDNA as a competitor, the level of transcript from the tester DNA template decreased drastically (Fig. 4C), indicating that TFID had been effectively sequestered by this competitor. In the control experiment, no inhibition of tester transcription occurred with pBR322 DNA which does not bind TFID specifically. These experiments showed that TFID does bind with rDNA template prior to and without TFIA and RNA polymerase I. The

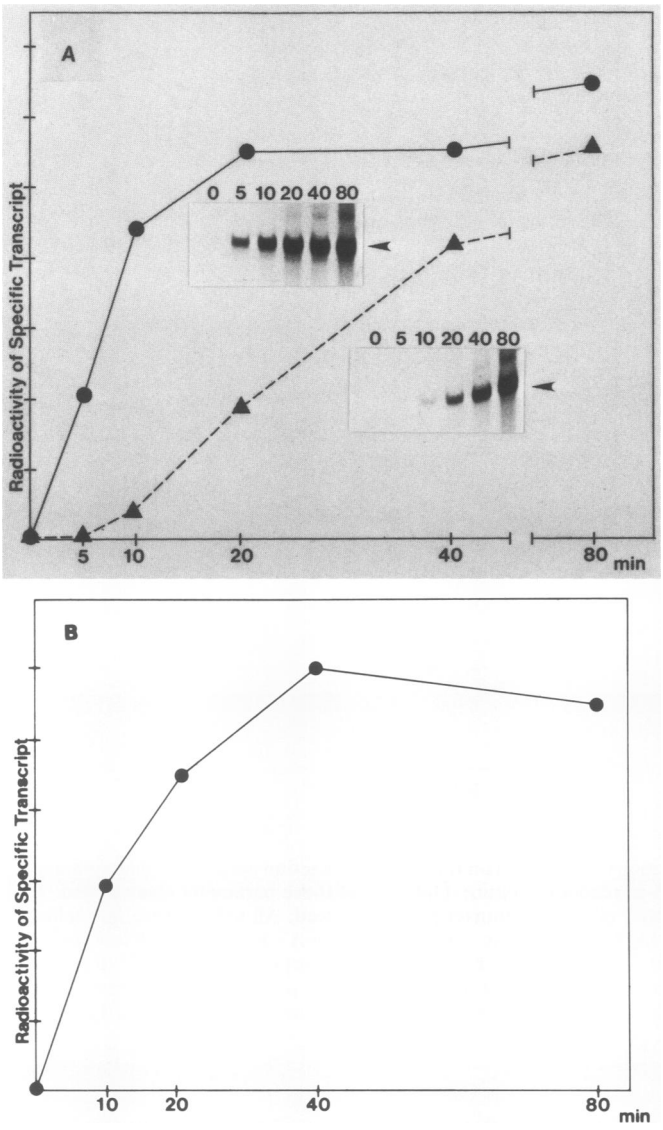

FIG. 3. Demonstration of the time required for the formation of the initiation complex. (A) Time course of incorporation of labeled nucleoside triphosphates into the accurate transcript with and without preincubation. Transcription reactions were started by the addition of four nucleoside triphosphates with (●) or without (▲) preincubation under standard conditions containing *Sal*I-cut pBE2Δ. The insets show the gel pattern at each time point. (B) Effect of preincubation time on the pulse-labeling of transcript. Fractions A′, P, and D were incubated with rDNA template for the indicated periods (abscissa) and then pulsed for 2 min by the addition of nucleotides, followed by a 3-min chase. The radioactivity in the correct transcript on the gel is plotted on the ordinate.

factor(s) in fraction A′ is referred to as TFIA, although we are aware that it may not be a single protein (see Discussion). Also, fraction P (partially purified RNA polymerase I) may contain some other factor(s). The second step, effective interaction of TFIA or RNA polymerase I or both to the template, requires the presence of TFID that is complexed with rDNA. The quantitative differences seen in Fig. 4B (lane 1 versus lanes 3, 4, and 7) may be due either to the time lag required for binding of TFIA and fraction P to form an active initiation complex on the rDNA-TFID complex or to the cooperation in binding among these factors. Further purification of fraction D with DEAE-cellulose and heparin agarose followed by phosphocellulose could concentrate TFID activity 10^4-fold (compared with S-100) without splitting the activity into multiple fractions (data will be published elsewhere). The purified fraction D exhibited the same characteristics with respect to complementation with other fractions and binding order.

Binding of TFIA and RNA polymerase I to the rDNA promoter requires the prior binding of TFID. To see whether TFIA and RNA polymerase I actually bind with the rDNA-TFID complex and to investigate the order in which binding occurs, we carried out another sequestration-type experiment in which the factors and the polymerase were preincubated with different templates and then short-pulsed after being mixed. It should be noted that in these experiments, a smaller amount of TFIA than in the experiments described in the previous section was used and that sequestration was shown to have occurred. TFIA or RNA polymerase I, or both, actually formed a complex together with template DNA which had been bound with TFID, because at least one of these molecules was not transferred from the template-factor-polymerase complex to the newly added templates having TFID, as evidenced by the absence of transcript on these templates (Fig. 5A, lanes 1 and 2). Lanes 3 and 4 suggest that some interaction between the RNA polymerase I and the rDNA-TFID complex exists which prevents the transfer of the polymerase to the newly added templates and that addition of TFIA to this state leads to the formation of the initiation complex. TFIA also formed a complex with the rDNA-TFID complex and did not move to the newly added template bound with TFID (lanes 5 and 6). Comparison of lanes 5 and 6 with lanes 3 and 4 clearly indicates that the initiation complex was formed at about a threefold higher level when TFIA was added first. These findings suggest that the formation of the initiation complex via the rDNA-TFID-TFIA complex is more efficient than via the rDNA-TFID-RNA polymerase complex under these conditions. Neither RNA polymerase I nor TFIA could be sequestered efficiently with template rDNA when the DNA was not bound with TFID (Fig. 5B). An indifferent DNA, pBR322, also could not sequester the enzyme (lane 6). From these observations, we conclude that the specific and stable binding of TFIA and RNA polymerase I to the rDNA template requires prior binding of TFID.

Resistance of the transcription complexes to Sarkosyl. To further analyze the initiation reaction in more detail, we took advantage of the effect of Sarkosyl on the transcription complexes, as had been studied in other systems (1, 14, 36). In this experiment, different concentrations of Sarkosyl as tested by Hawley and Roeder (14) to an RNA polymerase II system were added to the incubation mixture at various steps of formation of the transcription complex, and the amount of the complex was assayed by the accurate transcripts on the gel. The sensitivity to the detergent was distinctly different before and after the formation of the

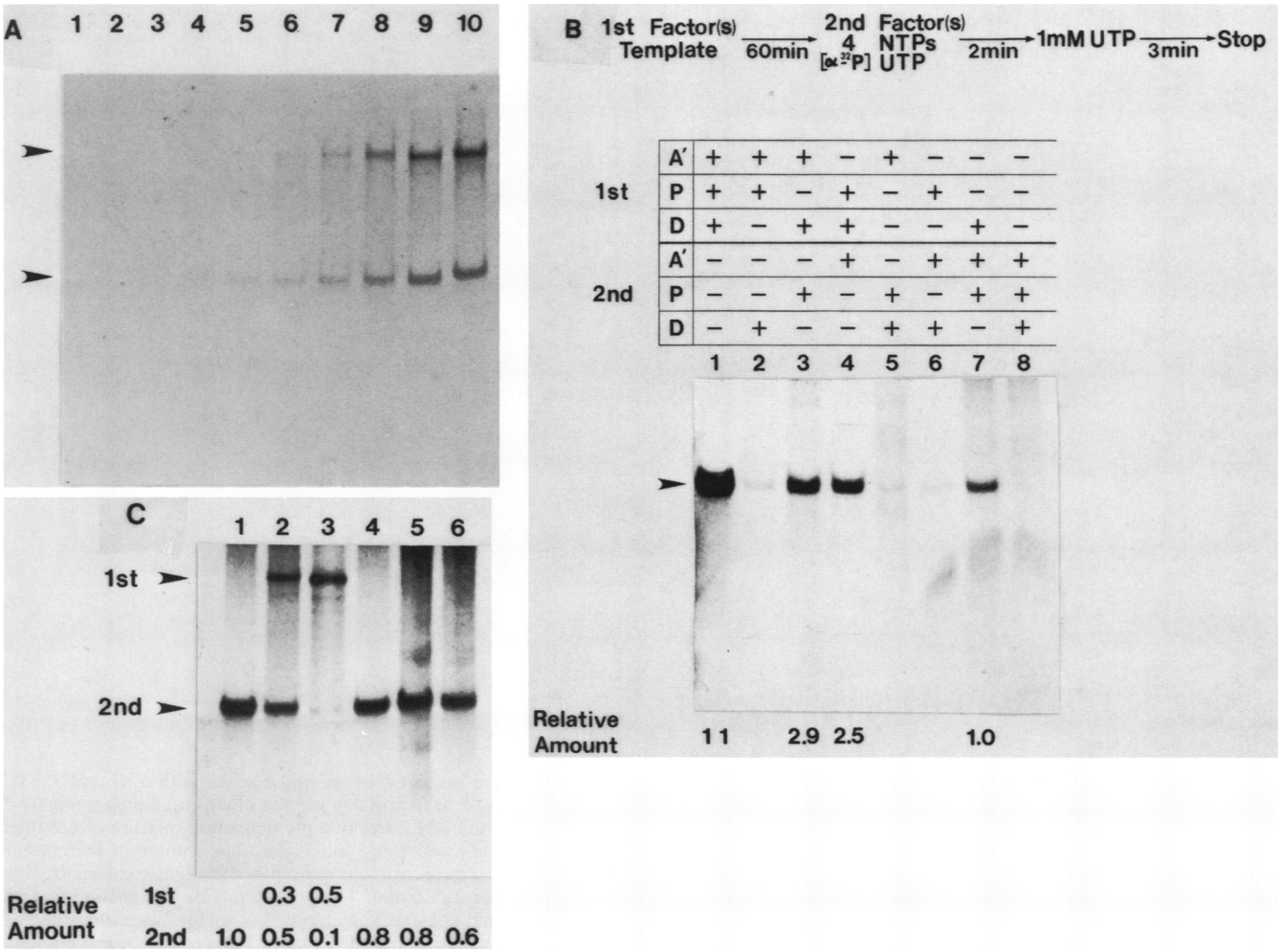

FIG. 4. Determination of the first binding factor to the template. (A) Determination of the elongation rate by RNA polymerase I in vitro. Two templates truncated at positions +696 and +2111 were incubated together (at an equimoler concentration) for 30 min in the presence of fractions A′, P, and D at 30°C. Elongation was started by addition of four nucleoside triphosphates. Samples were taken at 30-s intervals up to 120 s (lanes 1 to 5) and 1-min intervals up to 8 min (lanes 6 to 10, respectively), gel electrophoresed, and autoradiographed. (B) Preincubation and short-pulse experiments with different combination of fractions. Preincubation with the first factor(s) and template DNA for 60 min was followed by the addition of the second factor(s) and four nucleoside triphosphates, including [α-^{32}P]UTP. After the pulse incubation for 2 min, 1 mM unlabeled UTP was added and the elongation was allowed to proceed for 3 min. The amount of each component was the same as that in the standard mixture. The relative amount of each transcript was determined by densitometric tracing and shown under the lane. (C) Binding of a factor in fraction D to the template. The first (competitor) template was incubated with fraction D for 30 min at 30°C in a 20-μl mixture, to which the second (tester) template, fraction A′, and P fraction were added in that order, and incubated for another 30 min at 30°C. The mixture was then pulsed for 2 min as described for panel B. Lanes 1 to 3 contained, during the first preincubation, 0, 5, and 15 μl of *Pvu*II-cut pBE2Δ per ml, and lanes 4 to 6 contained the same amounts of *Eco*RI-cut pBR322. The template added for the second preincubation was 10 μg of *Sal*I-cut pBE2Δ per ml.

(pre)initiation complex (Fig. 6). Although 0.005% Sarkosyl did not interfere appreciably with complex formation and subsequent transcription initiation, 0.015 to 0.02% Sarkosyl almost completely inhibited the transcription when added before the complex was formed. Since this concentration of Sarkosyl did not inhibit transcription when the template was preincubated with the factors, we regard this inhibition as being due to the block of complex formation rather than the transcription initiation reaction. The step which was blocked by this concentration of Sarkosyl was found to be the binding of TFID, the first step, since preincubation of the template with fraction D alone before the addition of Sarkosyl resulted in the formation of active initiation complex by further addition of other factors in the presence of 0.02% Sarkosyl (Fig. 6, inset). This means that once TFID is bound to the template, 0.02% Sarkosyl cannot prevent the formation of the final initiation complex with other factors. A higher concentration of Sarkosyl rather sharply inhibited the transcription initiation by preformed complex, complete inhibition being obtained by 0.03 to 0.05% Sarkosyl. The mechanism of this inhibition may be explained in two ways. One is that the complex is dissociated at this Sarkosyl concentration, and the other is that, although the rDNA-TFID complex can survive 0.05% Sarkosyl, RNA polymerase I and TFIA cannot initiate transcription on the complex at this detergent concentration. Therefore, an experiment was carried out in which the template-factor-polymerase complex was exposed to 0.05% Sarkosyl once for 10 min and the short-pulse reaction was started after the detergent concentration had been decreased (by dilution) to 0.02%.

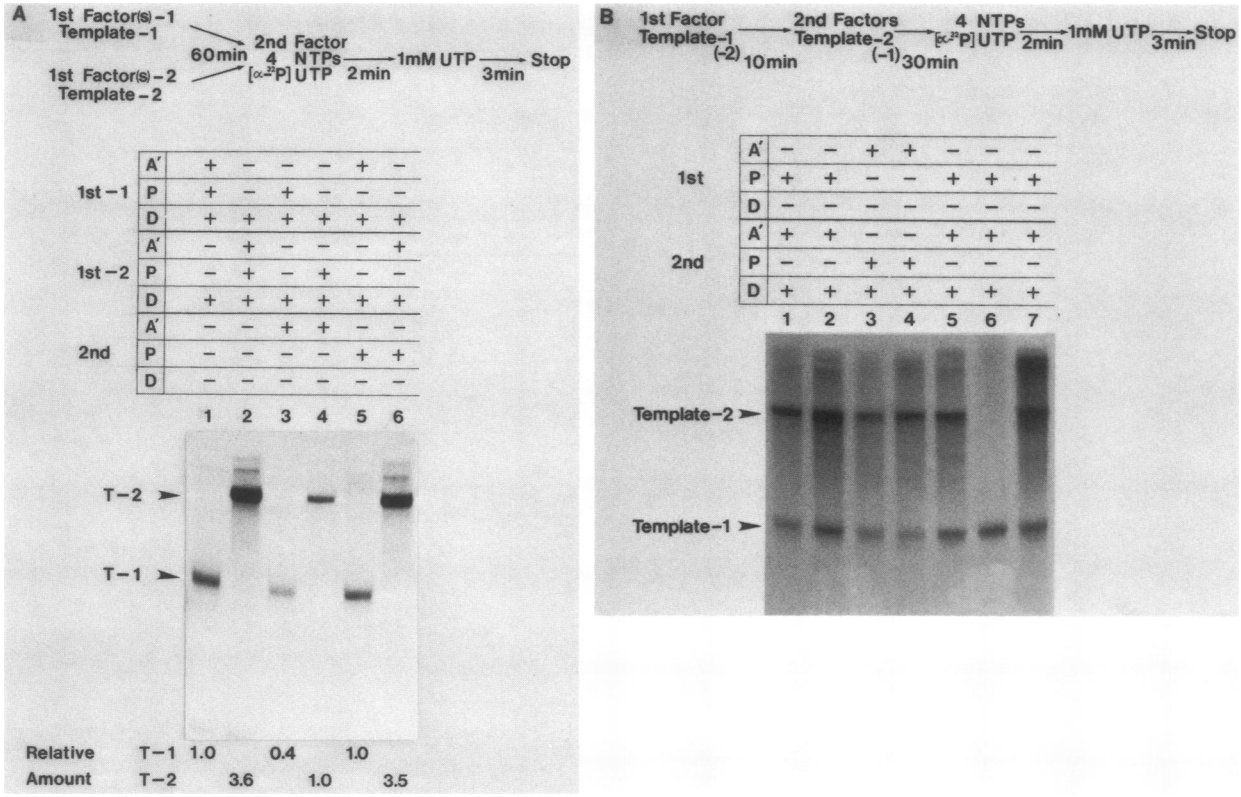

FIG. 5. Further analysis of binding order of A′ and P. (A) Templates 1 and 2 were prepared by cutting pBE2Δ with *Sal*I and *Pvu*II, respectively. Two preincubation mixtures were prepared, one containing template 1 and first factor(s) 1 and the other containing template 2 and first factor(s) 2. Preincubation was carried out under standard conditions for 60 min at 30°C. The two preincubation mixtures were then mixed, added with the last remaining factors and substrates, and short-pulsed for 2 min followed by a 3-min chase. The amount of each factor was half that in standard mixture for complete sequestration. The relative amount of each transcript was determined by densitometric tracing and is shown under the lane. (B) Template 1 (or 2) was preincubated with the first factor for 10 min. The other template and second factors were added to the mixture. After incubated for 30 min, the mixture was short-pulsed and chased. The amounts of factors were the same as those in standard mixture except for the following factors: lanes 1 and 2, 2 μl of P; lanes 3 and 4, 1 μl of A′; lanes 5 and 6, 1 μl of P. Template 1 was added first, and template 2 was added second in lanes 1 and 3; they were added in reverse order in lanes 2, 4, 5, and 7. In lane 6, the same amount of linearized pBR322 was added first and template 1 was added second.

There was little incorporation into accurate transcript under these conditions, indicating that rDNA-TFID complex had been either dissociated or irreversibly inactivated by 0.05% Sarkosyl (data not shown).

Conversion of the preinitiation complex to the initiation complex and kinetic evidence for possible reinitiation. We then examined the effect of nucleoside triphosphates as substrates on the stability of the initiation complex since it is known that in other RNA polymerase systems some of the nucleoside triphosphates at the start site stabilize the initiation complex and confer resistance to Sarkosyl (14, 34). The complex formed with human rDNA and HeLa fractions could generate the accurate transcript in the presence of 0.2% Sarkosyl only when both GTP and CTP were added previously to the mixture (Fig. 7). The fact that the first two nucleotides of the start site of the human rDNA are GC (10) and only this combination could protect the initiation complex from destruction by 0.2% Sarkosyl strongly suggests that a stable initiation complex is formed at the start site of rDNA with participation of the first two nucleoside triphosphates, GTP and CTP. When mouse rDNA and FM3A fractions were used for complex formation (Fig. 7), the complex became resistant only by the combination of ATP and CTP, the first two nucleotides at the start site of mouse rDNA (23). The reason why the band is so low in intensity may be explained by the one round of transcription that occurs under these conditions (see below). A faint band was visible in the presence of CTP, probably because we could not completely remove endogenous ATP. The third nucleotide of both human and mouse rDNA is T; therefore, the involvement of the third nucleotide may be ruled out. From above observations, we designate this more resistant complex the initiation complex, distinct from the preinitiation complexes formed only with factors and RNA polymerase I without these two nucleotides.

Experiments shown in Fig. 6 revealed that the rDNA-TFID complex with or without TFIA and RNA polymerase I was resistant to 0.02% but not to 0.05% Sarkosyl. Since the initiation complex was stabilized by GTP and CTP is resistant to 0.2% Sarkosyl (Fig. 7), we examined whether this complex can reinitiate transcription under the same detergent concentration. Figure 8 shows the time course of the synthesis of correct transcript determined by the gel. In the presence of 0.02% Sarkosyl, in which, once formed, the rDNA-TFID complex was stable but no new complexes formed, the incorporation into the transcript increased almost linearly without lag until about 30 min. The simplest explanation for this relatively long and almost perfectly linear incorporation is that reinitiation by RNA polymerase I is occurring under these conditions (note that it takes ap-

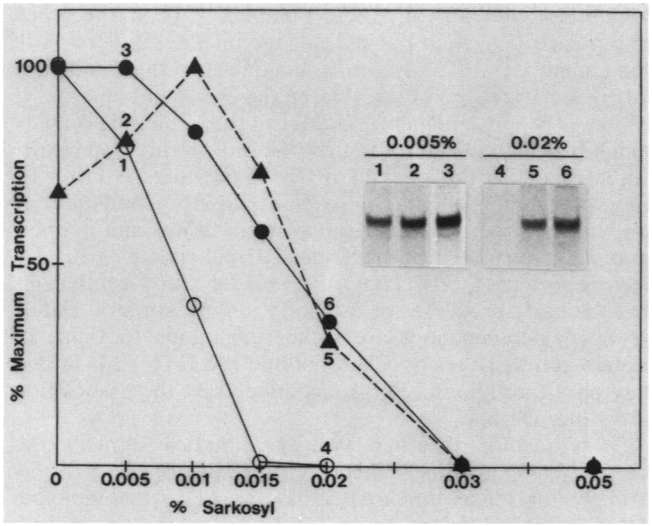

FIG. 6. Effect of different concentrations of Sarkosyl on transcription initiation. rDNA template (*Sal*I-cut pBE2Δ) was preincubated with fractions A′, P, and D for 60 min at 30°C in the presence of the indicated concentrations of Sarkosyl, and the transcription was continued for 10 min after the addition of nucleoside triphosphates (○). The same procedure was followed, except that Sarkosyl had been added after preincubation at the concentrations indicated on the abscissa (●). Preincubation was performed with fraction D alone, and the remaining factors and Sarkosyl at the indicated concentrations were added immediately before the start of transcription (▲). Inset: Parts of the actual gels are shown. Lanes 1 to 3 and 4 to 6 were incubated with 0.005 and 0.02% Sarkosyl, respectively. The numbers of the lanes from which the data are obtained are presented beside the marks on the gel.

proximately 1.5 min for one round of transcription of this template), although another possibility, that this increase of transcripts is only due to the time-dependent and well-balanced recruitment of preformed initiation complexes, cannot be ruled out completely. By contrast, in the presence of 0.2% Sarkosyl, incorporation stopped within 3 min, suggesting that essentially no reinitiation had occurred. These kinetics also suggest that the elongation rate is not decreased significantly under these conditions. These observations are consistent with the lability of the preinitiation complexes to 0.05% Sarkosyl and suggest that the initiation complex could not be reformed in the presence of this and higher concentrations of Sarkosyl.

DISCUSSION

The present study demonstrates the presence of multiple steps for the formation of initiation complex in rDNA transcription. The following picture emerges (Fig. 9). The rDNA promoter region first binds a species-dependent factor, TFID, to form the rDNA-TFID complex which is designated the preinitiation complex 1 (PIC-1). The exact site of TFID binding is not known. However, it is almost certain that the site lies on or close to the promoter region of rDNA (22; M. Nagamine et al., submitted for publication). Then, a factor(s) in fraction A′ (referred to as TFIA in this paper) comes to associate to PIC-1 to form the next preinitiation complex (rDNA-TFID-TFIA), named PIC-2. Then, RNA polymerase I binds with PIC-2 to make a quaternary complex (rDNA-TFID-TFIA-RNA polymerase I), designated preinitiation complex 3 (PIC-3). RNA polymerase I also interacts with PIC-1, even without TFIA (PIC-2′ in Fig. 9) and can form PIC-3 on addition of TFIA. Although the formation of PIC-3 via PIC-2 is more efficient than via PIC-2′ under the present experimental conditions, it is not certain which pathway is the one used in vivo. The incorporation of the first two nucleoside triphosphates, GTP and CTP in the case of human rDNA and ATP and CTP in the case of mouse rDNA, into this complex stabilizes it, forming the final initiation complex (IC), which is now ready to start with the addition of further nucleoside triphosphates (Fig. 9).

It is noteworthy that the formation of PIC-1 requires a relatively long time (Fig. 3). This may be due either to the very low concentration of TFID in this system or to the time-consuming reaction which takes place after binding. The finding that the TFID exists in extremely low amounts in the cell (unpublished observation) together with the kinetics of formation of initiation complex suggests that this factor may have an important role in the control of rDNA activation in vivo.

The use of different concentrations of Sarkosyl provided further information on the nature of complexes formed at different stages. The resistance curves to Sarkosyl (Fig. 6) are somewhat similar to those found for the RNA polymerase II-adenovirus major late promoter system (14). There is some difference, however, as regards the steps at which 0.015 or 0.02% Sarkosyl interferes. In the RNA polymerase

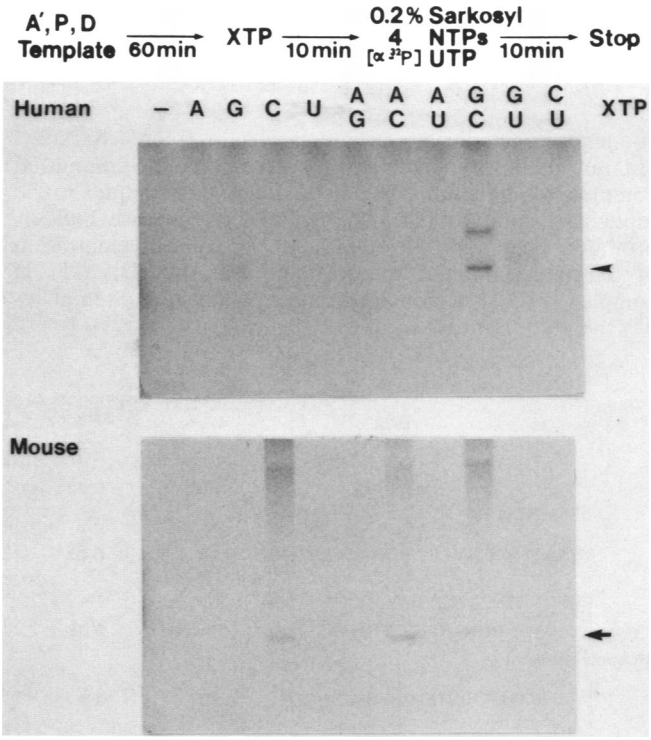

FIG. 7. Stabilization of initiation complex against 0.2% Sarkosyl by the first two nucleotides. (Top) The mixture contained fractions A′, P, and D from HeLa cells in adition to the human rDNA template. The indicated nucleoside triphosphate(s) were added to the reaction mixture after 60 min of preincubation. At 10 min later, Sarkosyl were added to 0.2% and elongation was allowed to proceed for 10 min by incubating with four nucleoside triphosphates. The resulting product is indicated by the arrowhead. (Bottom) The mixture contained fractions A′, P, and D from FM3A cells and the mouse rDNA template pMrSP harboring the *Sal*I(−167)−*Pvu*II (+291) rDNA fragment in the pBR322 vector. The reaction conditions were the same as in panel A. The accurately initiated 291-nucleotide transcript is shown by the arrow.

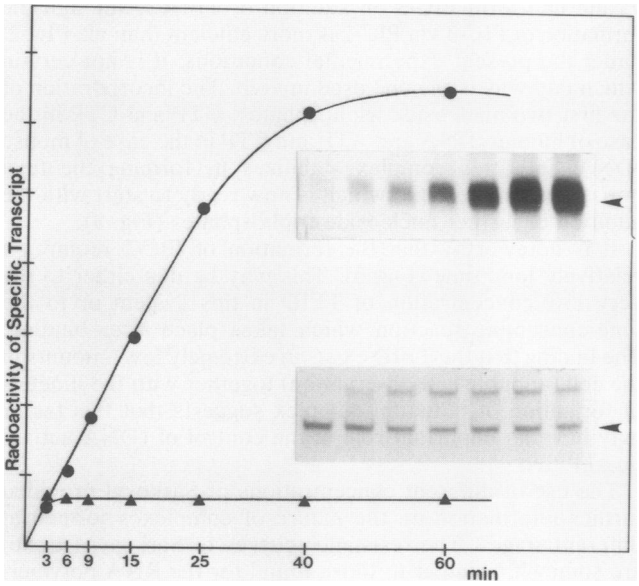

FIG. 8. Presence of prolonged transcription at 0.02% Sarkosyl. After preincubation under the standard conditions, Sarkosyl (0.02% [●]; 0.2% [▲]) and four nucleoside triphosphates were added to the mixture. In the case of 0.2% Sarkosyl, GTP and CTP were included in the preincubation mixture to stabilize the complex. The reactions were stopped at the indicated times, and the radioactivity incorporated into the correct transcript was determined by densitometric tracing.

II-adenovirus major late promoter system, 0.015% Sarkosyl did not inhibit the formation of the template-committed complex but did inhibit the conversion of this complex to the rapid-start complex (14). In the RNA polymerase I-rDNA promoter system, on the other hand, the same concentration of Sarkosyl inhibited the formation of the rDNA-TFID complex (PIC-1), which appears equivalent to the template-committed complex or stable complex (11) in the RNA polymerase II system but did not inhibit the conversion of this complex to PIC-3 which is analogous to the rapid-start (14) or activated complex (11) in the RNA polymerase II system. The much higher resistance of the initiation complex which had incorporated the first two nucleotides was quite similar between the systems of RNA polymerase I and II. The stabilization of the *Escherichia coli* RNA polymerase-template complex to higher salt concentrations and temperatures by the initiating nucleoside triphosphates has also been reported (4, 34). Thus, the present study establishes that the basic processes of transcription initiation on rDNA by RNA polymerase I are rather analogous to those of protein-coding genes by RNA polymerase II (11, 14) in that they pass through multiple steps defined by the association of distinct factors.

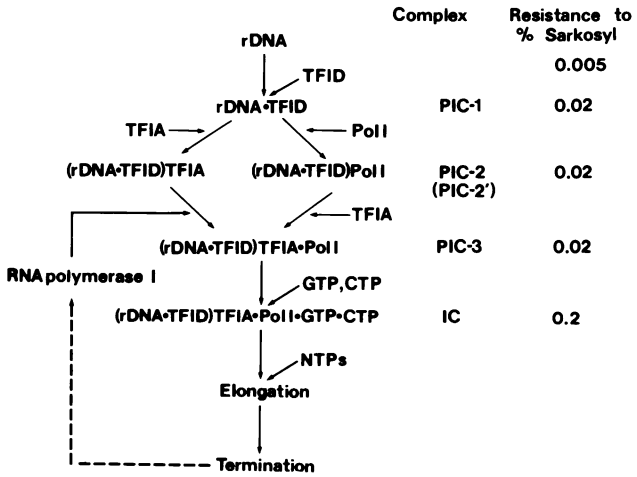

FIG. 9. Schematic representation of the formation of the transcription initiation complex on rDNA. Symbols: rDNA, ribosomal RNA gene fragment containing the promoter region; PolI, RNA polymerase I; PIC, preinitiation complex; IC, initiation complex; NTPs, nucleoside triphosphates as substrates. TFID and TFIA are defined in the text. Formation of PIC-3 via PIC-2 is more efficient than via PIC-2′ and may represent the in vivo pathway. The dotted pathway has not been rigorously proven.

These results, together with the kinetics showing the relatively long and linear incorporation seen in Fig. 8, argue strongly for the notion that TFID bound to the template rDNA remains there, while RNA polymerase I repeats transcription initiation, a scheme proposed by others (5, 38). Rigorous proof, however, must await further study with more purified components. In the present study, another factor, TFIA, appears to remain bound to the template. Although it is natural to postulate a ternary complex between the promoter region and two factors, it also remains to be proven. Since the binding of TFID appears to activate the rDNA promoter, it is reminiscent of the 40-kilodalton protein (TFIIIA) for the 5S RNA gene and the TATA box binding protein (TFIID) for some protein-coding genes (9, 29, 31). The function of TFIA is even more ambiguous, although it is definitely required for an accurate and efficient transcription of rDNA by RNA polymerase I aided by TFID (Fig. 2A and 4B). The former may help RNA polymerase I to bind to the promoter region together with TFID. Although only two factors are identified in this study, it does not exclude the possibility that more factors are involved in the transcription initiation on rDNA template, because all the fractions used in this analysis are only partially purified. TFID is likely to be a single protein or a tightly bound complex, judging from the data obtained on further purification. The TFIA fraction, however, contains a large amount of various proteins, and the activity of this fraction may be borne by two or more proteins. The fact that slight excess of this fraction already saturates the transcription (Fig. 2A) suggests that the factor(s) in this fraction is not rate limiting even in vivo. Fraction P, although partially purified with respect to RNA polymerase I, may also contain some factor which acts together with the enzyme. Further purification of each factor and reconstitution of the system with more purified fractions are required to clarify these points, including the precise stoichiometry between the factors and the template.

ACKNOWLEDGMENTS

We thank the members of this department for helpful discussion and Akira Ishihama, National Institute of Genetics, for critical reading of the manuscript.

This work was supported in part by grants from the Ministry of Education, Science and Culture and from the Foundation for Promotion of Cancer Research, Japan.

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