Assembly and Disassembly of the \textit{Drosophila} RNA Polymerase II Complex during Transcription*

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Initiation of mRNA synthesis by RNA polymerase II occurs by a multistep pathway that involves the polymerase along with several auxiliary factors. Here I describe an analysis of the kinetics and mechanism of action of \textit{Drosophila} RNA polymerase II by using the detergent Sarkosyl to inhibit different steps in the pathway leading to transcription initiation. The template DNA and \textit{Drosophila} transcription factors assemble into a partially Sarkosyl-resistant initiation complex with a kinetically first order time course characterized by a $t_1$ of about 3 min, and subsequent conversion of the initiation complex into the elongating complex occurs rapidly (<5 s). During the formation of the first two phosphodiester bonds, there is a stepwise transition from the initiation complex to the 0.25% Sarkosyl-resistant elongating polymerase. In contrast to the mammalian RNA polymerase II systems, the \textit{Drosophila} extracts possess the ability to carry out many rounds (~10) of transcription \textit{in vitro}. Finally, the transcription factors are committed to the template DNA as an initiation complex, but then no longer appear to remain committed to the DNA after initiation of transcription. This finding suggests that there is complete assembly and disassembly of the transcription complex during each round of transcription by RNA polymerase II.

Synthesis of mRNA by RNA polymerase II is a fundamental and important biological process. RNA polymerase II transcribes thousands of different genes, and its activity on these diverse genes must be accurately controlled. Purified RNA polymerase II is a multisubunit enzyme that can nonspecifically catalyze the synthesis of RNA from a DNA template, but it does not possess the ability to initiate transcription accurately and efficiently from a promoter. There are several auxiliary factors, which are generally referred to as TFIIA, TFIIB, TFIID, TFIIE, and TFIIH, that are required along with RNA polymerase II for accurate initiation at a promoter (for recent review, see Saltzman and Weikmann, 1989). In addition, RNA polymerase II activity is selectively controlled by a variety of trans-acting sequence-specific factors that bind to specific cis-acting sites in promoter and enhancer elements (for recent review, see Mitchell and Tjian, 1989). To understand the basic mechanisms by which RNA polymerase II recognizes promoters and is regulated by the sequence-specific factors, it will be necessary to elucidate the biochemical properties of the general RNA polymerase II transcription system that comprises RNA polymerase II and the auxiliary factors.

There are a variety of experimental approaches to the study of the RNA polymerase II transcription machinery. For instance, the polymerase and auxiliary factors can be fractionated and purified. These experiments would lead to the identification and characterization of the auxiliary factors. Alternatively, the RNA polymerase itself can be studied in detail, and these investigations would increase our understanding of the central enzyme involved in transcription. On the other hand, it is also possible to characterize the properties of the RNA polymerase II apparatus as a whole by treating the multicomponent system as a complex enzyme. For example, the formation of intermediate complexes can be monitored by the use of agents that inhibit different steps in the transcription process. In studies on mammalian RNA polymerase II transcription, reagents that have been used for this purpose include Sarkosyl, an anionic detergent (Gariglio et al., 1974; Ackerman et al., 1983; Tolnay et al., 1984; Hawley and Roeder, 1985, 1987), heparin, an anionic polymer (Reinberg and Roeder, 1987), poly(3-deoxyinosinic-3-deoxyctydyl)ic acid (Fie et al., 1984), and a mixture of ammonium sulfate and manganese(II) chloride (Safer et al., 1985).

In parallel with studies on the purification and characterization of the general RNA polymerase II transcriptional machinery from \textit{Drosophila} embryos, I have employed the detergent Sarkosyl to characterize various steps in the transcription process by \textit{Drosophila} RNA polymerase II. The \textit{Drosophila} embryo transcription system is attractive to study not only because it is highly active (Soeller et al., 1988; Heiermann and Pongs, 1985), but also because it will enable biochemical analysis of transcriptional regulatory events that are important for the proper development of the embryo to be carried out. Furthermore, the available data on \textit{Drosophila} transcription indicate that the mechanism of RNA polymerase II transcription in \textit{Drosophila} is similar to that in mammalian cells. For instance, some \textit{Drosophila} and \textit{HeLa} factors are functionally interchangeable \textit{in vitro} (Heberlein et al., 1985), and mammalian genes can be accurately transcribed \textit{in vitro} with \textit{Drosophila} embryo extracts and vice versa (Heiermann and Pongs, 1985). The high activity of \textit{Drosophila} embryo extracts has also enabled transcriptional activation by the rat glucocorticoid receptor to be achieved \textit{in vitro} (Freedman et al., 1989). Moreover, by comparison with cultured cells, \textit{Drosophila} embryos are an inexpensive source of transcription factors. Thus, studies on the RNA polymerase II transcription system from \textit{Drosophila} embryos should re-

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veal many important features of the basic transcription process in eukaryotes. In this work, I describe the characterization of the crude RNA polymerase II transcription system from Drosophila embryos. In most of these experiments, I used the promoter of the Drosophila gap gene Krüppel (Rosenberg et al., 1986; Gaul and Jäckle, 1987), which is a strong promoter that directs accurate transcription in vitro. By using the anionic detergent Sarkosyl, I have been able to distinguish different steps in the pathway leading to transcription initiation as well as to follow the activity of transcription factors through multiple rounds of transcription.

**EXPERIMENTAL PROCEDURES**

**Materials—**Ribonucleoside 5'-triphosphates and deoxyribonucleoside 5' triphosphates were purchased from Pharmacia LKB Biotechnology Inc. (ultrapure fast protein liquid chromatography-purified grade). Avian myeloblastosis virus reverse transcriptase was from Life Sciences. Sarkosyl, actinomycin D, and Hepes were obtained from Sigma. The KCl was from Riedel De Haen. Carrier yeast RNA (≥0.1 mg/ml) was from Boehringer Mannheim. Carrier yeast RNA was prepared by exhaustive extraction of crude Torula yeast RNA (Sigma) with phenol and then with chloroform followed by dialysis to remove traces of residual chloroform. The synthetic oligonucleotides that were used in the primer extension experiments of the Krüppel and hunchback transcripts were prepared by Operon Technology.

Plasmids and Synthetic Oligonucleotides—Krüppel DNA was obtained from Michael Hoch and Dr. Herbert Jäckle (University of Munich) as pHBXS, which contains a 1.3-kb SalI to XbaI fragment of the Krüppel gene that encompasses the region from −861 to +426 relative to the transcription start site (Gaul and Jäckle, 1986). This 1.3-kb SalI-XbaI fragment was subcloned into SalI and XbaI sites in the poly linker of pUC119 to give pKr, which was used in most of the in vitro transcription experiments. The plasmid pKr-mut is identical with pKr except that it contains the sequence (given in the same sense as the Krüppel transcript) 3' GGTGACTCGACTTACAGGATCACCTGGCC 5' inserted downstream of the promoter between the positions corresponding to +18 and +19 of the Krüppel transcript. The plasmid pHPP1 contains the 0.3-kb HindIII to EcoRI fragment of the hunchback gene, which was obtained from Jon Margolis and Dr. James Posakony (University of California, San Diego), inserted downstream of the promoter from −698 to −281 relative to the RNA start site (Tautz et al., 1987). The primer for reverse transcription analysis of the Krüppel RNA, named Kr3, is 5' TATTTACTCGGTTTGGTGTGCAACAC 3' and hybridizes to the pKr transcript from +45 to +72 relative to the start site and to the pKr-mut transcript from +74 to +101. The primer for reverse transcription analysis of the hunchback RNA, named hbP1, is 5' GTGCGCTGCTCTGCTGAATGGCGCGGTGCTTTC 3' and hybridizes to the 3.2-kb hunchback distal (P1) transcript from +25 to +53.

In Vitro Transcription and Primer Extension Analysis—All experiments were carried out with a minimum of two independent times to ensure reproducibility of the data. Drosophila embryo extracts were prepared essentially as described by Soeller et al. (1988) with Canton S embryos collected between 0 and 12 h after fertilization. The reaction medium for a standard in vitro transcription experiment contained 32.5 mM Hepes, K', pH 7.6, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM dithiothreitol, 0.05 mM EDTA, 5% (v/v) glycerol, 0.5 mM in each of 4 deoxyribonucleoside triphosphates, 20 µg/ml supercoiled plasmid DNA, and Drosophila embryo extract to 5 µg/ml protein concentration in a final volume of 25 µl. In reactions that included Sarkosyl, 2 µl of a Sarkosyl solution that was 12.5 times the desired final concentration was added to a 25-µl reaction. The reaction mixtures were carried out at 21 °C for the indicated times and were stopped by the addition of 20 mM EDTA, pH 8.0, 2 mM NaCl, 1% (w/v) sodium dodecyl sulfate, and 0.25 mg/ml carrier yeast RNA (100 µl). Then, 2.5 mg/ml proteinase K (5 µl) was added, and the mixture was incubated at room temperature for 5 min. Next, 0.3 M NaOAc (500 µl) was added, and the solution was extracted once with phenol/

chloroform (400 µl; 1:1, v/v) and once with chloroform/isomyl alcohol (400 µl; 24:1, v/v). The resulting aqueous phase (400 µl) was combined with 0.30 ml of 5'-32P-labeled primer (1 µl of 0.03 pmol/µl), and the nucleic acids were precipitated with ethanol (1 ml). The pellet was dissolved in 0.3 M NaOAc (200 µl), and the nucleic acids were reprecipitated with ethanol (500 µl). The labeled transcript was digested with 75% (v/v) ethanol, dried in a SpeedVac concentrator, and dissolved in 2 mM Tris-HCl, pH 7.8, 0.2 mM EDTA, 0.25 mM KCl (10 µl). This solution was subsequently incubated at 58 °C for 1 h to allow hybridization of the primer to the transcripts and then cooled to room temperature. Then, a solution containing 20 mM Tris-HCl, pH 8.7, 10 mM MgCl₂, 100 µg/ml actinomycin D, 5 mM dithiothreitol, 0.33 mM in each of 4 deoxyribonucleoside 5'-triphosphates, and 10 units of avian myeloblastosis virus reverse transcriptase (25 µl) was added, and the resulting mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of ethanol (300 µl). The nucleic acids were precipitated, washed with 75% (v/v) ethanol, dried in the SpeedVac concentrator, and dissolved in formamide loading buffer, 0.1 M NaOH (9 µl; 2:1, v/v). The solution was heated to 90 °C for 3 min and then applied to a polyacrylamide-urea gel.

**RESULTS AND DISCUSSION**

The Use of Sarkosyl to Demarcate Distinct Steps in the Transcription Process—To test the possibility that various concentrations of Sarkosyl can be used with a crude Drosophila embryo extract to inhibit different steps in the transcription process, the scheme outlined in Fig. 1, which had been previously used in studies on mammalian RNA polymerase II (Hawley and Roeder, 1985, 1987), was followed. For these experiments, the promoter of the Drosophila gap gene Krüppel (Rosenberg et al., 1986; Gaul and Jäckle, 1987) was used. Transcription of the Krüppel promoter initiates at multiple sites, and this strong promoter is accurately transcribed in vitro. In the first series of in vitro transcription reactions, varying concentrations of Sarkosyl were added to the template DNA before addition of transcription factors. The mixtures were run on agarose gels, and autoradiograms were obtained of the gels, which were either wet or dried. The autoradiograms were scanned with an LKB Ultrascan densitometer.

**FIG. 1. Different concentrations of Sarkosyl inhibit distinct steps in the pathway leading to transcription initiation.** Incubations were carried out at 21 °C with 20 µg/ml pKr DNA as outlined in the scheme (see text for details). The indicated concentrations of Sarkosyl or water (as a control) were added at time 0 (lanes 1–5), (lanes 7–12), or (lanes 13–18). The primer extension products of the transcripts are shown.
were incubated for 60 min to allow initiation complex formation, and ribonucleoside triphosphates were then added to initiate transcription (Fig. 1, lanes 1–6; Sarkosyl #1). In the second set of reactions, varying amounts of Sarkosyl were added to preformed initiation complexes, and nucleotides were subsequently added to initiate transcription (Fig. 1, lanes 7–12; Sarkosyl #2). In the third series, nucleotides were added to preformed initiation complexes, and then varying levels of Sarkosyl were added to the elongating polymerase (Fig. 1, lanes 13–18; Sarkosyl #3). These experiments show that Sarkosyl at a concentration of 0.02% (w/v) or greater will effectively inhibit assembly of the initiation complex, whereas levels of Sarkosyl between 0.02% and 0.06% (w/v) will inhibit initiation complex formation, but allow the conversion of the initiation complex to the elongating enzyme. At the highest Sarkosyl concentration used in this study (0.25%), there was no detectable inhibition of the elongating polymerase. Hence, Sarkosyl can be successfully employed in transcription reactions with Drosophila embryo extracts to inhibit formation of the initiation complex, yet enable the complex, upon addition of nucleotides, to carry out one round of transcription. Note also that the lower level of transcription in Fig. 1, lanes 8–10 and 14–18, relative to lanes 1, 7, and 13, is due to multiple rounds of transcription in lanes 1, 7, and 13, and a single round of transcription in lanes 8–10 and 14–18.

**Determination of the Rate-limiting Step in the Pathway Leading to Initiation**—An important property of a transcription system is the kinetics of the steps in the pathway leading to initiation. Thus far, the transcription process in *Drosophila* has been separated into the assembly of an initiation complex, which occurs in the absence of nucleotides, followed by conversion of the complex into the 0.25% Sarkosyl-resistant intermediate by the addition of nucleotides. To measure the rate of assembly of the Drosophila RNA polymerase II initiation complex, the scheme outlined in Fig. 2A was followed. The template DNA and transcription factors were combined and incubated at 21°C to allow formation of initiation complexes. After a designated period of time, 0.05% Sarkosyl was added to inhibit further complex assembly, and then nucleotides were added to initiate transcription. The results of a typical experiment are shown in Fig. 2A. The data from three independent experiments were fitted to an exponential curve to give a $t_{1/2}$ of 3.2 ± 1.3 min (90% confidence limits; Fig. 2B). As control experiments, it was also demonstrated that initiation complex assembly does not occur at 0°C (incubation on ice; Fig. 2A, compare lanes 10 and 11), and that the samples are inert at room temperature for 30 min after addition of the stop solution (Fig. 2A, compare lanes 10 and 12).

The rate of conversion of the initiation complex to the 0.25% Sarkosyl-resistant intermediate was then determined. In these experiments, nucleotides were added to preformed initiation complexes to begin transcription, and, after a designated period of time, Sarkosyl was added to 0.25% concentration, which is sufficiently high to inhibit transcription from the initiation complexes. These studies revealed that within 5 s after addition of nucleotides, the initiation complexes are quantitatively converted into the 0.25% Sarkosyl-resistant intermediate (Fig. 3, lanes 1–4). As a control, 0.25% Sarkosyl was added to the initiation complexes before the nucleotides, and, consistent with the results in Fig. 1, there is complete inhibition of transcription (Fig. 3, lane 6). Thus, in a manner similar to the HeLa RNA polymerase II system (Hawley and Roeder, 1985, 1987), the assembly of the initiation complex is the rate-limiting step in the transcription reaction. The rate of initiation complex formation in the *Drosophila* system ($t_{1/2} = 3.2$ min) is, however, more rapid than that of the mammalian system ($t_{1/2} = 9.0$ min, Hawley and Roeder, 1985; $t_{1/2} = 8$ min, Hawley and Roeder, 1987). This difference may be an important factor in the higher levels of transcription obtained with the *Drosophila* transcription system relative to the mammalian systems.

**Nucleotide Requirements for Formation of the 0.25% Sarkosyl-resistant Intermediate**—To understand more precisely the conversion of the initiation complex to the 0.25% Sarkosyl-resistant form, the ribonucleoside triphosphate requirements for this process were examined. The *Krüppel* transcript initiates at two major sites, which are designated +1 and +5.
While the +1 transcript should not initiate. On the other hand, in the presence of UTP + ATP, the first two bonds of the +1 transcript should form while the +5 transcript should not initiate. Using this approach, the effect of no nucleotides (as a control), ATP alone, ATP + GTP (to form one phosphodiester bond at +5), or UTP + ATP (to form two bonds at +1) upon preformed initiation complexes was examined. Following the scheme depicted in Fig. 4, some, but not all, nucleotides were added to initiation complexes. Varying concentrations of Sarkosyl were added 20 s later, and then all four ribonucleoside triphosphates were added 20 s after the Sarkosyl to permit transcription elongation.

These experiments revealed that resistance to Sarkosyl increases with the number of phosphodiester bonds that are formed. The initiation complex, in the absence of nucleotides, is resistant to 0.05% Sarkosyl, and, thus, the reactions containing 0.05% Sarkosyl represent one round of transcription (Fig. 4, lanes 2, 7, 12, and 17). Then, as the Sarkosyl concentration was increased to 0.25%, there was partial resistance upon formation of the first AG bond at +5 (Fig. 4, compare lanes 12 and 14) and nearly complete resistance upon formation of the first two bonds at +1 (Fig. 4, compare lanes 17 and 19).

In these experiments, it is sometimes difficult to eliminate the possibility that contaminating nucleotides have altered the observed findings. For example, the low level of 0.25% Sarkosyl-resistant transcription in the presence of ATP alone could be due to a trace of GTP in the Drosophila extract (Fig. 4, lane 9). Because of the multiple Kriippel RNA start sites, however, there are internal controls for contaminating nucleotides in the reactions containing ATP + GTP and UTP + ATP. For example, if contaminating UTP were present in the ATP + GTP reactions (this would enable formation of the trinucleotide, AGU, beginning at +5), then transcription initiation would be observed at +1 in addition to +5. Since initiation does not occur at +1 as well as +5 in the ATP + GTP reactions (Fig. 4, lanes 12–14), it is reasonable to conclude that they did not contain significant levels of contaminating UTP. In addition, reactions were performed in which the transcription stop solution was added 20 s after addition of the incomplete nucleotides (Fig. 4, lanes 5, 10, 15, and 20). These control reactions ensured that a significant level of background RNA synthesis had not occurred before addition of the Sarkosyl.

In studies using mammalian transcription factors with the adenovirus major late promoter, which begins with the sequence 5’ ACUCUCUCCG, there are differing reports with regard to the conversion of the initiation complex to the Sarkosyl-resistant intermediate. With the HeLa transcription system, Hawley and Roeder (1985) as well as Arias and Dynan (1989) found that ATP + CTP are necessary and sufficient to generate a 0.4% Sarkosyl-resistant intermediate. These findings suggest that formation of the first phosphodiester bond is the key step in the conversion of the initiation complex to the resistant form. In contrast, however, Conaway and Conaway (1988), using rat liver transcription factors with the same promoter, found that ATP + CTP + UTP, but not ATP + CTP, were sufficient for generation of the 0.25% Sarkosyl-resistant form. The data described here with the Drosophila system are somewhat intermediate between the previous findings and suggest a gradual, rather than a sharp, transition from the initiation complex to the 0.25% Sarkosyl-resistant elongating polymerase.

**Efficiency of Transcription by the Drosophila RNA Polymerase II System**—Two important parameters of the transcription reaction are the fraction of promoters that are produc-
tively transcribed and the number of rounds of transcription that can be obtained from each promoter. To determine the performance of the Drosophila in vitro transcription system, these parameters were measured by carrying out reactions either in the presence of Sarkosyl under conditions that allowed only one round of RNA synthesis or in the absence of Sarkosyl to yield multiple rounds of transcription. The results from a typical experiment are shown in Fig. 5. When Sarkosyl is employed to limit RNA synthesis to one round, roughly 3% of the DNA templates are transcribed (Fig. 5, lanes 2–6). On the other hand, when Sarkosyl is omitted from the reaction, there is up to a 10-fold increase in the level of transcription (Fig. 5, lanes 7–16). As a control, it was also demonstrated that no detectable RNA synthesis occurs when nucleoside triphosphates are omitted from the transcription reaction (Fig. 5, lanes 1 and 2). Thus, it appears that an estimated 3% of the DNA templates contain productive initiation complexes, and that about 10 rounds of transcription can be achieved with the Drosophila embryo transcription system.

Why would only 3% of the DNA templates contain productive initiation complexes? It is likely that an important factor in the inefficiency of in vitro transcription is the number of factors that are required for initiation. In mammalian systems, it is believed that six factors (RNA polymerase II, TFIIA, TFIIB, TFIID, TFIIE, and TFIIF) are necessary for transcription initiation (for review, see Saltzman and Weinmann, 1989). If each of the six factors were distributed randomly among the DNA templates at an efficiency of 50%, then only \( \frac{0.50^6}{6!} = 0.016 \), or 1.6%, of the promoters would contain a complete initiation complex. Other considerations include nonspecific inhibitors of transcription, such as RNases, DNases, nonspecific DNA binding proteins, and phosphatases.

In this work, transcription was observed to be linear through the first 60 min, and, during this period, roughly 3% of the templates undergo about 8 rounds of transcription each to give an overall efficiency of 0.2 transcript/template/h. This transcriptional efficiency is comparable to that previously reported with Drosophila transcription systems (Parker and Topol, 1984; Heiermann and Pongs, 1985) and is higher than the efficiencies of \( \leq 0.03 \) transcript/template/h that were observed with HeLa transcription systems (Weil et al., 1979; Manley et al., 1980; Dignam et al., 1983; Dynan and Tjian, 1983). It thus appears that the major difference between the performance of the Drosophila and HeLa factors is not the efficiency of transcription complex formation, but rather the number of rounds of transcription that can be attained in vitro. This conclusion is also consistent with the observation that the HeLa transcription system will undergo only about two rounds of transcription (Hawley and Roeder, 1987). At present, the Drosophila in vitro transcription system is not as well characterized as the HeLa transcription system, but, in the future, it may be possible to optimize conditions for the reactions as well as for the preparation of transcription factors to maximize the efficiency of in vitro RNA synthesis.

Commitment of Transcription Factors to the DNA Template—Since there were multiple rounds of transcription in vitro, I examined whether or not reinitiation preferentially occurs on the same DNA templates that were used in the initial round of transcription. In other words, does a stable complex form on the template DNA from which transcription could be reinitiated by a shorter and faster pathway than de novo assembly of the initiation complex from factors in solution? To address this question, initiation complexes were preferred on a first DNA template (DNA-1), a second template (DNA-2) was added under different conditions, and then the relative level of transcription was measured from the two templates. The template DNAs that were used in these experiments contained the wild-type Krüppel promoter (Kr), an insertion mutant of the Krüppel promoter (Kr-mut), or the upstream promoter of the Drosophila hunchback gene (hbP1; Tautz et al., 1987). The Kr-mut promoter, which is transcribed less efficiently than the Kr promoter, was used because its transcript, which is 29 nucleotides longer than that of the wild-type Krüppel transcript, could be monitored simultaneously with the Kr transcript by primer extension analysis.

First, to test the template commitment and stability of the initiation complexes, the following operations were carried out: 1) initiation complexes were assembled on DNA-1; 2) DNA-2 was added; 3) after 0, 1, 5, 10, 20, or 30 min, 0.05% Sarkosyl was added to inhibit new assembly of initiation complexes, but to permit transcription initiation and elongation from the preformed complexes; and 4) ribonucleoside triphosphates were added to initiate transcription (see scheme outlined in Fig. 6). Using combinations of either pKr and pKr-mut (Fig. 6A) or pKr and phbP1 (Fig. 6B), the initiation complexes formed on DNA-1 were found to be resistant to challenge by DNA-2 for at least 30 min. These experiments indicated that the initiation complex is inert to the presence of a second template in the absence of transcription. In addition, there is no detectable transcription from DNA-2 in these experiments, and, thus, at least one of the essential transcription components is stably and quantitatively depleted from the extract by either specific or nonspecific interaction with DNA-1.

Next, I examined the level of transcription initiation from DNA-2 relative to reinitiation of transcription from DNA-1 when DNA-2 and ribonucleoside triphosphates are added simultaneously to preformed initiation complexes on DNA-1 (see scheme outlined in Fig. 7). If an activated complex that survives multiple rounds of initiation were formed on DNA-
1, then there would be preferential transcription from DNA-1 relative to DNA-2. In a manner parallel to the previous template commitment experiments (Fig. 6), combinations of either pKr and pKr-mut (Fig. 7A) or pKr and phbP1 (Fig. 7B) were used. The initiation complexes were committed to DNA-1 through the first round of transcription (Fig. 7A, lanes 1, 2, 7, and 8; Fig. 7B, lanes 1, 2, 7, and 8), but, beginning with the next round of initiation, the template selection was random (Fig. 7A, lanes 3–6, 9–12; Fig. 7B, lanes 3–6, 9–12). By determining the relative levels of transcription from pKr and pKr-mut as DNA-1 or DNA-2, there was no preferential transcription of DNA-1 over DNA-2 after the first round of RNA synthesis on DNA-1 (Fig. 7, C and D). These results demonstrate that the bulk of the template-committed transcription factors are no longer committed to DNA-1. Since only about 3% of the DNA-1 templates contain productive initiation complexes, however, it is necessary to address whether or not the few productive transcription complexes present in the reaction also disassemble. If stable complexes remained on DNA-1 while the bulk of the factors were released into solution, the newly formed complexes would be expected to be distributed randomly between DNA-1 and DNA-2, which would result in a higher rate of transcription from DNA-1 (which would contain productive transcription complexes from round #1 plus the newly formed round #2 complexes) compared with DNA-2 (which would contain only the newly formed round #2 complexes). However, preferential transcription of DNA-1 relative to DNA-2 is not observed after the first round of transcription, and, hence, it appears that stable, activated complexes are not formed on DNA-1. Furthermore, it is likely that the addition of nucleotides to the template-committed initiation complexes releases the promoter-bound factors by a mechanism similar to the bulk of the template-committed transcription factors. Thus, the new findings suggest that there is complete assembly and disassembly of transcription complexes during each round of RNA synthesis.

Previous studies with HeLa transcription factors have addressed questions similar to those that have led to these experiments but report contradictory data (Safer et al., 1985; Hawley and Roeder, 1987). Using two variants of the adenovirus major late promoter, Hawley and Roeder (1987) reported a 3-fold preference for transcription from the first template upon which the initiation complexes were originally formed relative to the second template that was added later, but they also did not observe depletion of transcription factors from their extracts by the first template. Consequently, in their experiments, soluble factors were available for assembly of initiation complexes on the second template even in the absence of transcription. Safer et al. (1985), also using two variants of the major late promoter, found commitment to the first template in the absence, but not the presence, of active transcription. They did not, however, find commitment to the first template through the first round of transcription. The observations of Hawley and Roeder (1985) might be due to the presence of the sequence-specific transcriptional activator, USF/MLTF (Sawadogo and Roeder, 1985; Carthew et al., 1985), in their extracts. It is possible that this sequence-specific DNA binding factor was preferentially bound to the major late promoter in first DNA template and that it was responsible for the difference in transcription from the first and second templates. In these studies with the Krüppel promoter, we have not found any sequence-specific transcription factors analogous to USF/MLTF that activate transcription from the promoter region. Consequently, the experiments probably reflect the properties of a minimal promoter in the absence of additional influence by factors other than the basic RNA polymerase II machinery.

A Proposed Model for RNA Polymerase II Transcription—Based upon the findings in this study with the in vitro transcription system from Drosophila embryos, I have constructed a simple model for RNA polymerase II transcription, which is depicted in Fig. 8. Briefly, soluble transcription factors and template assembly into an initiation complex,
FIG. 7. Disassembly of template-committed transcription factors after initiation of transcription. Initiation complexes were assembled at 21 °C with DNA-1 for 10 min, and a mixture of DNA-2 and ribonucleoside triphosphates was added (DNA-1 and DNA-2 were each at a concentration of 20 μg/ml). At the indicated times, 0.05% Sarkosyl was added to inhibit further complex formation. The primer extension products of the transcripts, which are indicated by brackets, are shown. A, pKr = DNA-1, pKr-mut = DNA-2 (lanes 1–6), and pKr-mut = DNA-1, pKr = DNA-2 (lanes 7–12). B, pKr = DNA-1, phbP1 = DNA-2 (lanes 1–6), and phbP1 = DNA-1, pKr = DNA-2 (lanes 7–12). C, levels of pKr transcript synthesized when pKr is added either as DNA-1 (open squares) or as DNA-2 (open circles). The value of 1.0 corresponds to the amount of transcript synthesized in a single round of transcription when pKr is added as DNA-1. D, levels of pKr-mut transcript synthesized when pKr-mut is added either as DNA-1 (solid squares) or as DNA-2 (solid circles). The value of 1.0 corresponds to the amount of pKr-mut transcript synthesized in one round of transcription when pKr-mut is added as DNA-1.

which is committed to the DNA template, by apparent first order kinetics with a $t_{1/2}$ of about 3 min. During the synthesis of the first couple of phosphodiester bonds, the transcription complex is rapidly converted (<5 s) into a 0.25% Sarkosyl-resistant elongating enzyme. Meanwhile, the complex at the promoter has disassembled, and the next round of transcription begins by reassembly of the new initiation complex from the factors in solution.

Comparison of Drosophila and HeLa RNA Polymerase II Transcriptional Machinery—As previously noted, the Drosophila and HeLa RNA polymerase II transcription systems are functionally interchangeable, and, consequently, they are basically similar. In both Drosophila and mammals, there is a slow assembly of the initiation complex followed by a rapid conversion to the elongating enzyme. In Drosophila extracts, however, the rate of formation of the initiation complex ($t_{1/2}$
~ 3 min) is significantly faster than that observed with HeLa cell factors (t1/2 ~ 8–9 min; Hawley and Roeder, 1985, 1987). In addition, it is possible to obtain up to 10 rounds of transcription using Drosophila extracts, whereas HeLa factors will yield only about 2 rounds of transcription (Hawley and Roeder, 1987). Given the apparent requirement for complete assembly of the initiation complex for every round of transcription, these two parameters (t1/2 of complex formation and number of rounds of transcription) are likely to be related.

To examine the requirement for hydrolysis of the β-γ bond of ATP at the onset of transcription initiation, which has been well documented in the HeLa transcription system (Bunick et al., 1982; Sawadogo and Roeder, 1984; Arias and Dyman, 1989), experiments were carried out using the ATP analogues dATP (which can be hydrolyzed at the β-γ position, but is not incorporated into the RNA) and AMP-P(NH)P (which cannot be hydrolyzed at the β-γ position, but can be incorporated into RNA). These studies revealed that, in contrast to the HeLa system, dATP is efficiently incorporated into the RNA and that AMP-P(NH)P inhibits transcription by the Drosophila RNA polymerase II machinery (data not shown). The use of dinucleotides as primers for transcription initiation, which had been previously observed with HeLa RNA polymerase II (Samuels et al., 1984), was also investigated. In these experiments, however, neither UpA nor ApG was able to serve as primers for initiation at the +1 and +5 positions of the Krüppel transcript (data not shown).

**Function of Sequence-specific Transcriptional Activators—** RNA polymerase II transcription is regulated by sequence-specific transcriptional activators that bind to selected promoter and enhancer elements (for a recent review, see Mitchell and Tjian, 1989). The experimental approach used in this work can also be applied to the study of these factors. For instance, do the sequence-specific factors affect the assembly of initiation complexes? To address this question, we could measure the rate of complex assembly in the presence or absence of sequence-specific activators as well as the effect of the activators upon the efficiency of formation of productive initiation complexes. Furthermore, we could begin to examine if the sequence-specific factors are committed to the DNA template for multiple rounds of transcription. In this manner, we can understand the biochemical function of the regulatory transcriptional activators within the framework of the basic mechanism of RNA polymerase II transcription.

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