Transcription initiation by RNA polymerase II from the adenovirus major late promoter was studied in vitro in a nuclear extract derived from HeLa cells. We found that different concentrations of Sarkosyl blocked transcription initiation at several different functional steps. These corresponded to two steps defined previously in a partially purified transcription system: formation of the rapid start complex, which required incubation of template with the extract and was blocked by Sarkosyl concentrations >0.025%, and productive initiation, which occurred upon addition of nucleotides and was prevented by Sarkosyl concentrations >0.1%. We used assays based on these findings to prove further the events that comprise the processes of initiation and reinitiation of transcription from the major late promoter. We found that the templates used in the first round of initiations from preformed rapid start complexes were more actively transcribed in the second round of initiations than newly added templates. We argued that the most likely explanation for this observation was that some transcription component(s) remained committed to the promoter after formation of an elongation complex. This template-committed complex must have been distinct from the rapid start complex, because reinitiation and rapid start complex formation were both blocked by 0.025% Sarkosyl.

We previously demonstrated that transcription initiation from the adenovirus major late promoter could be separated into three functional steps in vitro (1). These steps were defined on the basis of successive changes in the sensitivity of the initiation reaction to Sarkosyl upon incubation of the promoter with the protein fractions required to reconstitute transcription. For example, an incubation at 30°C in the absence of nucleotides was both necessary and sufficient to confer resistance to Sarkosyl concentrations in the range of 0.015-0.02%. We called the intermediate formed during this incubation the "rapid start complex" because it was capable of initiating transcription within 30 s after addition of nucleoside triphosphates. Once formed, the rapid start complex was inhibited from producing a transcript if Sarkosyl was added to a concentration of 0.05% before the addition of nucleotides. We argued that the step necessary to confer resistance to 0.05% Sarkosyl was probably formation of the first phosphodiester bond, because only the appropriate combination of two nucleotides, those corresponding to the first two nucleotides incorporated into the RNA, would result in conversion from Sarkosyl sensitivity to Sarkosyl resistance. However, we will refer to this step as productive initiation to include the possibility that elongation beyond the first phosphodiester bond was required to convert the rapid start complex to an elongation complex capable of completing a transcript in the presence of concentrations of Sarkosyl >0.05%.

The RNA polymerase II transcription system we used previously was reconstituted with protein fractions prepared from HeLa cell nuclear extracts by chromatography on phosphocellulose. We chose to use a partially purified system initially to eliminate possible complexities resulting from the presence of endogenous nucleotides. In this study, we have tested the applicability of these methods to unfraccionated nuclear extracts.

There were two main incentives for developing the methodology necessary for defining discrete functional steps in the initiation mechanism in a transcription system reconstituted from an unfraccionated extract. First, most other laboratories studying RNA polymerase II transcription in general or the expression in vitro of a specific class II promoter use nuclear or whole cell extracts. Yet many aspects of these transcription systems have never been adequately characterized. Most of the effort directed toward optimizing conditions in vitro has focused on obtaining the maximum signal or the greatest difference in signal without an understanding of what reaction parameters have been affected by the change in reaction conditions. Such an approach can result in uncertainty about whether the observed behavior in vitro is of physiological relevance. An increased knowledge about the nuclear extract system will therefore be useful both to those wishing to use nuclear extracts in vitro and also to those seeking to interpret published information obtained using nuclear extracts. Second, it has been demonstrated that transcription from the major late promoter requires at least five proteins in addition to RNA polymerase II and that other promoters use additional factors not required by the major late promoter (2-12). The ability to obtain mechanistic information in the unfraccionated system, even if this information is quite limited, could be valuable in assessing whether all of the factors involved in transcribing a particular promoter in vitro are present in a transcription system reconstituted from purified components.

In this paper, we describe our analysis of functional steps in transcription initiation from the major late promoter in the unfraccionated nuclear extract. We have found conditions that allow measurement of the rate and extent of reinitiation of transcription and have used this method to show that
Steps in Initiation and Reinitiation by RNA Polymerase II

Fig. 2. Concentrations of Sarkosyl required to inhibit two steps in transcription initiation in vitro. Sarkosyl was added to individual transcription reactions at two different times, indicated by the letters a and b. For each reaction, major late promoter-containing plasmid DNA (pMLH2) was incubated with nuclear extract for 60 min at 30 °C in a total volume of 20 μl, using the standard conditions described under “Experimental Procedures.” For the a reactions (O), Sarkosyl at the concentrations shown was present during the initial incubation. For the b reactions (C), Sarkosyl was added to the indicated concentrations following the initial incubation and 30 s before addition of nucleoside triphosphates (NTP’s). Additional Sarkosyl was added to all reactions to a final concentration of 0.08% and 0.1% 30 s after nucleotide addition. The reactions, in a final volume of 30 μl, were incubated at 30 °C for an additional 45 min. The transcription reactions were processed and subjected to polyacrylamide gel electrophoresis as described under “Experimental Procedures.” The 306-nucleotide run-off transcripts were excised from the gel and the amount of transcript was determined as described under “Experimental Procedures.”

transcription in this system remains committed to the major late promoter for more than one round of initiation events.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Sarkosyl Titrations**—The concentrations of Sarkosyl required to inhibit transcription initiation from the major late promoter in a nuclear extract system were determined in the experiment of Fig. 2. The protocol followed is summarized schematically below the figure. The DNA was incubated with the nuclear extract for 60 min at 30 °C. Sarkosyl, at the concentrations shown in the figure, was present either during the entire incubation (curve a) or only during the last 30 s of the incubation (curve b). Nucleoside triphosphates were then added, followed 30 s later by addition of Sarkosyl to 0.08%. The reaction was stopped 45 min after nucleotide addition and the amount of transcript produced in each reaction was measured following gel electrophoresis of the reaction products.

The second addition of Sarkosyl was included in the experimental protocol to prevent reinitiation from occurring in all reactions, even those in which the amount of Sarkosyl already present in the reaction was not sufficiently high to limit transcription to a single round of initiations. This method simplified interpretation of the data and allowed a direct determination of the concentrations of Sarkosyl required to inhibit formation of the rapid start complex (curve a) and productive initiation of transcription (curve b). The concentrations of Sarkosyl required for inhibition of both steps in nuclear extracts were somewhat higher than those required in the fractionated system. For example, complete inhibition of rapid start complex formation required 0.025% Sarkosyl in the nuclear extract, but only 0.015–0.02% in the fractionated system. Similarly, when Sarkosyl was added to the reaction after rapid start complex formation, the amount of Sarkosyl required for complete inhibition was shifted from 0.05% in the fractionated system to 0.1% in the nuclear extract.

We previously reported that concentrations of Sarkosyl greater than or equal to 0.05%, when added after nucleotides, interfered with production of full-length transcripts from the major late promoter and caused the accumulation of transcripts that were prematurely paused or terminated about 186 nucleotides downstream of the cap site (1). This behavior was also observed with the nuclear extract, although the effective concentration of Sarkosyl was found to be shifted to around 0.1% (data not shown). To eliminate this complexity, we have constructed a plasmid containing the major late promoter but lacking the downstream sequences we have found to be required for the production of the prematurely terminated transcripts (see “Experimental Procedures;” Fig. 1). This plasmid, pMLH2, was the template used in most of the experiments described in this paper, including that of Fig. 2. The production of a full-length 306-nucleotide transcript from this template was unaffected by the inclusion of up to 0.6% Sarkosyl during the elongation phase of the reaction (data not shown).

**The Effect of Nucleotide Concentrations on Initiation**—In a system reconstituted with fractions, we had found that ATP and CTP must be added to preformed rapid start complexes in order to pass the step blocked by 0.05% Sarkosyl (1). Other combinations of nucleotides, including ATP and UTP or any nucleotide added singly to the reaction, failed to result in Sarkosyl resistance. The simplest explanation for this observation was that Sarkosyl resistance required minimally the formation of the first phosphodiester bond, since the major late RNA begins pppACUCU . . . .

The nuclear extract contains small amounts of nucleotides and, therefore, there was a possibility that initiation of transcription could occur without added nucleotides. However, the experiment of Fig. 2 showed that addition of nucleotides was required to confer resistance to 0.1% Sarkosyl, indicating that the concentrations of endogenous nucleoside triphosphates were not sufficient to support productive initiation. This result led us to examine the nucleotide concentration requirements for initiation as well as the rate of degradation of nucleotides in our extracts to determine whether rapid depletion of nucleotides could lead to a decreasing rate of transcription initiation.

In the experiment of Fig. 3, which is described in detail in the Miniprint Supplement, we found that all four nucleoside triphosphates were rapidly hydrolyzed (t1/2 ~ 20 min) when added to the nuclear extract at concentrations standard for the in vitro transcription assays. The addition of phospho-
Nucleotides required for productive initiation from the major late promoter in the nuclear extract system

| Nucleotides          | % initiated complexes |
|----------------------|-----------------------|
| -NTPs                | <1                    |
| 0.6 mM ATP + CTP     | 100 (3.7)*            |
| 0.1 mM ATP + CTP     | 10.4                  |
| 0.6 mM ATP           | 1.4                   |
| 0.1 mM ATP           | 0                     |
| 0.6 mM CTP           | 2.3                   |
| 0.1 mM CTP           | <1                    |
| 0.6 mM UTP           | <1                    |
| 0.1 mM UTP           | <1                    |

*The amount of transcript measured in this reaction is an underestimate of the actual amount (see text).

The experiment of Fig. 3 had demonstrated that all four of the nucleoside triphosphates were degraded at the same rate in the nuclear extract. Therefore, the experiment of Fig. 4 did not address which nucleotide(s) limited transcription initiation. To examine this question directly, we measured the extent of productive initiation (resistance to 0.1% Sarkosyl) in the presence of nucleoside triphosphates added individually or in combinations of two. The results are shown in Table II. Nucleotides at the concentrations shown were incubated with the extract and the template for 50 min at 30 °C. Phosphocreatine was included in half of the reactions, as indicated in the table. After the initial incubation, Sarkosyl was added to 0.1%, followed by addition of all four nucleoside triphosphates, and the reactions were incubated an additional 30 min to allow transcription production. The amount of transcript observed in each reaction was normalized to that produced in the reaction to which 0.6 mM ATP and CTP were added (conditions that would have resulted in initiation from all rapid start complexes in the fractionated system). An amount of initiated transcript >1% was visible as a distinct band on the autoradiogram. Productive initiation did not occur when nucleotides were omitted, even in the presence of phosphocreatine. However, the addition of ATP, CTP, or UTP alone produced some stably initiated complexes when phosphocreatine was also present during the incubation. The ability of 0.6 mM CTP to produce the greatest number of initiated complexes was consistent with an independent determination of the concentrations of ATP and CTP required for productive initiation from the major late promoter in the fractionated system, which indicated that the apparent Km for CTP was about 10-fold higher than that for ATP. The fact that each
of the nucleotides was able to promote productive initiation in the presence of phosphocreatine indicated that the other nucleotides were present in the extract, presumably in the form of di- and/or monophosphates. (The preparations of nucleotides used were high performance liquid chromatography purified and were shown not to contain contaminating nucleoside triphosphates by their inability to promote productive initiation in any but the expected combinations in the fractionated system. However, we cannot rule out the possibility that the nucleotides contained contaminating amounts of the nucleoside di- and monophosphates. This possibility does not alter the main conclusion of this experiment, however.)

In the presence of phosphocreatine, 0.6 mM ATP and CTP appeared to result only in a low level of initiation. This result was misleading, because the low incorporation was actually the result of extensive elongation before addition of the labeled GTP to the reaction. We determined this fact directly by monitoring the decrease in label incorporated into the transcript with increasing time of incubation of rapid start complexes with ATP and CTP before addition of Sarkosyl and labeled nucleotides (data not shown).

**Extent of Rapid Start Complex Formation not Limited by Availability of Transcription Factors**—The time required to form rapid start complexes on the major late promoter was measured by adding promoter-containing DNA to nuclear extract and, at subsequent times, adding samples of this reaction to nucleotides and 0.03% Sarkosyl. The amount of transcript produced after an additional 30-min incubation was then determined and is shown in Fig. 5. The formation of rapid start complexes proceeded with apparent first order kinetics with a $t_d$ of 8 min. The reaction was apparently complete after 50–60 min; further incubation before nucleotide addition did not increase the number of complexes (data not shown).

In the course of these studies, we used three different major late promoter-containing plasmids (pMLH1, pMLH2, and pSmaf; see "Experimental Procedures") and found that although the rate of rapid start complex formation did not vary significantly among the plasmids, the extent of the reaction did. When we varied the DNA concentration and measured the amount of transcript produced in the presence of 0.025% Sarkosyl following a 60-min incubation of DNA with nuclear extract, we found that transcription increased with DNA concentration until a concentration of 35 µg/ml was used. At that and higher concentrations (up to at least 60 µg/ml), the amount of transcript remained constant. The lowest DNA concentration required for maximum transcription (35 µg/ml) was the same for all of the plasmids; however, the maximum level of transcription was approximately 2-fold higher for pMLH1 and pMLH2 than for the larger plasmid pSmaf.

It is important to note that the amount of transcription approached at most one or two transcripts produced for every 100 promoters added to the reaction, so that the number of promoters per se did not limit the maximum level of transcription.

One possible explanation for the similar dependence on total DNA concentration but different final level of transcription among the different plasmids was that the extent of rapid start complex formation was not limited by the quantitative binding of a limiting transcription factor but, rather, was determined by the outcome of a competition between complex formation and inactivation of the DNA template. We therefore wanted to determine whether the components of the reaction that were apparently "depleted" during complex formation were the transcription factors or the DNA. We incubated a plasmid containing the major late promoter (pMLH2) with nuclear extract for 60 min; then added a second major late promoter-containing plasmid (pMLH1) which produces a run-off transcript of a different size (diagrammed in Fig. 1). At various times following addition of the second template, we removed samples of the reaction and added 0.025% Sarkosyl and nucleotides, and allowed the preformed rapid start complexes to produce transcripts. The outcome, shown in Fig. 6, was that even though rapid start complex formation had gone to completion on the template present in the first 60-min incubation (open circles), rapid start complexes could still form on the template added subsequent to this first incubation (solid circles). Furthermore, these complexes did not form on the second template by depleting complexes from the first template, because the number of rapid start complexes committed to the first template did not decrease during the second incubation period. The results of this experiment conclusively demonstrated that the extent of rapid start complex formation was not limited by the availability of transcription factors. Since a second DNA was able to form rapid start complexes, some acquired property of the first DNA must have been responsible for limiting the reaction to the observed level.

The experiment of Fig. 6 also demonstrated the stability of the rapid start complexes to 0.025% Sarkosyl, even in the presence of additional template. When 0.025% Sarkosyl was included in the second incubation, there was no loss of rapid start complexes from the first DNA during the second 80-min incubation in either the absence (open squares) or presence (open triangles) of the second major late promoter-containing template. As expected, no rapid start complexes formed on the second template in the presence of Sarkosyl (solid triangles). The long lifetime of rapid start complexes was also

**Fig. 5. Time required for formation of rapid start complexes on the major late promoter.** To initiate the reactions shown, pMLH2 DNA was added to the nuclear extract in a volume of 220 µl. The DNA and extract were preincubated separately so that both were at 30 °C when the reaction was initiated; the solution conditions were as described under "Experimental Procedures." At the indicated times, 20-µl portions were removed and added to 0.04% Sarkosyl; 30 s later, nucleoside triphosphates were added and the reactions were incubated an additional 30 min at 30 °C. The final concentration of Sarkosyl was 0.028% in a final reaction volume of 27 µl. The fractional amount of transcript produced in each reaction (F) was determined by normalizing to the maximum amount of transcript observed (128 pm). The single exponential curve drawn through the points was fit to the early (F < 0.8) time-points; $t_d$ = 8 min.
Transcription from a second major late template after completion of rapid start complex formation on a first major late template. Real cut pMLH2 DNA (30 μg/ml) was incubated with nuclear extract for 60 min at 30 °C in a volume of 475 μl. This mixture was then split into three reactions, with 137.5 μl of the incubated mixture increased to 150 μl by addition of Sarkosyl to 0.025% (C), 4 μg of Smal cut pMLH1 DNA (C, •), or both the second DNA template and Sarkosyl! (Δ, A). At the indicated times following these additions, 19-μl portions of each reaction were removed and added to the four nucleoside triphosphates and the amount of Sarkosyl required to bring the final concentration to 0.025% in a total volume of 24 μl. These transcription reactions were incubated for 45 min at 30 °C. The quenched reactions were processed and analyzed by polyacrylamide gel electrophoresis as described under “Experimental Procedures.” The amounts of the run-off transcripts from the pMLH2 template (open symbols) and from the pMLH1 template (solid symbols) were determined and plotted versus the elapsed time of the second incubation. The horizontal line indicates the mean value for the amount of transcript produced from the pMLH2 (DNA1) template in all the reactions (43 pm). The line fitting the values obtained for the pMLH1 template (DNA2) in the reaction to which Sarkosyl was absent in the second incubation (●) is a single exponential curve approximating a maximum value of 18.6 pm with k = 9.9 min.

observed previously in the transcription system reconstituted with fractions.

Transcription Remains Committed to a Template for More than One Round of Initiations—Sarkosyl concentrations of 0.015% and 0.025% in the transcription systems reconstituted with fractions and nuclear extract, respectively, not only prevented formation of rapid start complexes, but also prevented reinitiation of transcription, demonstrating that the Sarkosyl-sensitive step must be repeated with each initiation event. However, it was not known whether all of the steps necessary for a first round of transcription must be repeated with each subsequent initiation, or whether some transcription factors remain associated with the promoter during transcription. A answer to this question led us to demonstrate that reinitiation had occurred and also to determine whether the reinitiations occurred on the same templates used in the first round of initiations.

Our strategy for addressing this problem is shown schematically in the lower part of Fig. 7. We allowed rapid start complex formation between the major later promoter and factors in the nuclear extract to go to completion. We then split the preincubated mixture into six reactions. To three of these reactions, we added 0.025% Sarkosyl so that only one round of transcription could occur. The other three reactions contained no Sarkosyl and transcription could reinitiate following the first round of transcription. We then followed the amount of transcript produced in a single round and in multiple rounds from reactions to which no second DNA was added (panel A), to which a second DNA containing the major late promoter was added (panel B), and to which vector DNA containing no bona fide class II promoter was added (panel C).

Comparison of the level of transcription observed in the presence and absence of Sarkosyl showed that reinitiation did occur in all of the reactions to which Sarkosyl was not added. Although neither the vector nor the second major late promoter-containing DNA significantly affected the amount of the transcript observed in one round of synthesis, both reduced, to the same extent, the amount of transcript observed in reactions in which transcription proceeded beyond one round of initiation. (Compare solid squares in Fig. 7, panel A, with those in panels B and C.) We concluded, therefore, that this reduction was neither dependent on nor significantly affected by the presence of the major late promoter in the second DNA. In addition, following the completion of the first round of transcription, the first template (panel B, solid squares) was transcribed approximately 3-fold better than the second major late template (panel B, open squares), although the concentrations of the two promoters were the same. (A low level of transcription of the second template was expected because, as described above, rapid start complexes could form on a second template in the absence of transcription and without loss of complexes from the first template under these conditions, which were essentially the same as those of Fig. 6.)

We have repeated these experiments with a higher concentration of DNA in the first incubation in an attempt to sequester the limiting transcription components before the addition of the second DNA. However, this approach had a technical limitation, in that high concentrations of template added subsequent to the first incubation depressed the level
of transcription to such an extent that it was not always possible to be certain that reinitiation had occurred. We have also tried reducing the amount of extract, instead of increasing the amount of template, but have not succeeded in finding conditions that will completely exclude formation of rapid start complexes on the second template, when assayed according to the protocol of Fig. 6. We therefore cannot rule out the possibility that some of the transcription of the second DNA was dependent on factors lost from the first template. However, as we have never seen an equal amount of transcription from the first and second added templates after the first round of transcription, we favor the conclusion that some transcription factor(s) tended to remain committed to the template at least through two rounds of transcription.

**Kinetics of Reinitiation**—In experiments where the accumulation of transcripts after addition of nucleotides to rapid start complexes was monitored (e.g. Figs. 4 and 6), we have consistently observed that after completion of one round of synthesis, continued synthesis proceeded apparently linearly but at a slower rate than that observed initially. One possible interpretation of this observation is that fewer rapid start complexes participated in the second round of synthesis than in the first round. A second possibility is that the time required to reform rapid start complexes was significant relative to the time required to elongate the transcripts to full length.

In the experiment of Fig. 7, when additional DNA was added to the reaction at the same time as nucleotides (panels B and C), the final rate of accumulation of transcript was the same as that observed when no additional DNA was added (panel A). However, the total amount of transcript produced in 75 min was lower in the reactions of panels B and C for one of two possible reasons. 1) The lower rate of synthesis was achieved earlier in the reaction (at about 5 min, as indicated by the extrapolated dotted lines in panels B and C), before the completion of one round of synthesis. 2) The reaction completed one round of synthesis normally but the entry of the reaction into the second round was delayed. Either interpretation is consistent with the experiment of Fig. 7, although the results of similar experiments (not shown) suggested that the first round of transcription was completed normally, while the length of the delay in beginning the second round was variable and was increased by increasing the final concentration of DNA added to the reaction.

In order to understand both the decreased rate of transcript accumulation beyond the level of one round and the effect of additional DNA on the kinetics of multiple round transcription, we directly measured the rate at which new rapid start complexes participated in the second round of synthesis. These small reactions were incubated an additional 30 min after Sarkosyl addition to allow full-length transcript production from all rapid start complexes. The amount of 306-nucleotide transcript from the pMLH2 template (open symbols) and 536-nucleotide transcript from the pMLH1 template (solid squares) was determined and is plotted as a function of time after addition of nucleotides. The arrow indicates the amount of transcript produced when Sarkosyl was added to 0.025% to a portion of the initial incubated reaction 30 s before nucleotides. This reaction contained the same components in the same concentrations as the other reactions and was treated identically in all respects other than order of addition of Sarkosyl and nucleotides.

Sarkosyl before nucleotides and incubating for 50 min as for the other reactions.

This experiment showed that addition of nucleotides was followed by a lag phase (at least 5 min) in which no new rapid start complexes were formed. When a second promoter-containing DNA was added with nucleotides, the length of this lag was increased but the kinetics of the subsequent reaction were essentially identical to those which followed the lag phase in the absence of a second DNA. As was also observed in the experiment of Fig. 7, the effect of the second DNA on the kinetics of the reaction was not dependent on the presence of the major late promoter, since the amount of transcript produced in the reactions to which pUC or pMLH1 DNA were added was similar at every time point. Although rapid start complexes did form on the pMLH1 template (solid...
squares), the number of these complexes was only about one-fifth the number of new complexes formed on the pMLH2 template (DNA1) after nucleotide addition. This result provided additional support for the conclusion that the DNA template used in the first round of RNA synthesis was transcribed in the second round in preference to newly added DNA.

The kinetics of initial formation of rapid start complexes and the reformation of complexes following nucleotide addition were clearly different, because no lag phase was observed when nuclear extract and the template were first combined (Fig. 5). These observations suggested that the transcripts initiated during the first round of synthesis must undergo some elongation before new rapid start complexes begin to form. This elongation could be necessary either to free the promoter for the binding of new transcription factors or to release some component of the reaction that must finish elongation before becoming available for the formation of new rapid start complexes (e.g., the polymerase). This second possibility seemed less likely because of the observation that the formation of rapid start complexes was not limited initially by the availability of transcription factors (Fig. 6). We have done preliminary experiments to test the effect of the elongation rate on the rate at which reformation of rapid start complexes occurred. We found similar lag periods for a variety of elongation rates (adjusted by varying the UTP concentration). For example, in one side by side comparison of two reactions, we found the same number of new rapid start complexes formed by 10 min after addition of nucleotides. A separate measurement of the elongation rates in these reactions showed that within 10 min all of the transcripts initiated in the first round had been fully elongated in one reaction, whereas less than 25% of the transcripts had been completed in the other reaction. This result strongly suggested that the completion of transcripts was not required for formation of new rapid start complexes, but left open the possibility that a step preceding formation of the rapid start complex, which was inhibited by 0.025% Sarkosyl, intervened in the effective concentration of a transcription factor due, for example, to nonspecific binding to DNA or to instability at 30 °C.

**DISCUSSION**

The experiments we have presented here showed that several functional steps in transcription initiation could be observed in a nuclear extract system using appropriate concentrations of Sarkosyl. Two steps apparently correspond to those observed previously with the more purified system, namely, formation of the rapid start complex and formation of a productively initiated complex. We had previously defined an additional step preceding formation of the rapid start complex and resulting in a template-committed complex (1). The ability to detect this complex relied on the finding that the inhibition of rapid start complex formation by 0.015% Sarkosyl was reversible when the Sarkosyl concentration was decreased by dilution following the initial incubation. We have not been able to repeat this result with the nuclear extract system, possibly because the higher concentration of Sarkosyl needed to inhibit rapid start complex formation (0.025%) irreversibly inactivated some component necessary for transcription. However, we have demonstrated a template committed complex as an intermediate in the reinitiation of transcription in the nuclear extract system. As the model in Fig. 9 illustrates, we propose that there is a continued association of some component of the transcription machinery at a step preceding formation of the rapid start complex, which was inhibited by 0.025% Sarkosyl in the second round of transcription initiation as well as the first.

The issue of which or not a template containing a class II promoter remains committed to transcription through multiple rounds of initiation events could not previously be resolved *in vitro*. In the RNA polymerase I and III systems, an unambiguous demonstration that transcription factors do remain bound to a promoter through multiple initiation events was possible because the total amount of transcription observed was greater than one transcript for every promoter present in the reaction, proving that reinitiation must have taken place; yet there was no loss of factors to competing templates (15–17). In contrast, both RNA polymerase II transcription systems (at least the unfractionated extracts) produce at most about one transcript for every 10 promoters in a standard reaction (14, 18). Without a way to block reinitiations, it was not possible to determine that they had occurred. (Reinitiation is defined here as an initiation event that could not take place until after a previous initiation event.) The experiments presented here and in our previous publication (1) demonstrated that reinitiations were occurring in our transcription systems and, furthermore, showed that the level of transcription without reinitiations could be determined. We used this technique to show that when transcription was allowed to continue after preformed rapid start complexes had initiated, the template on which these rapid start complexes had been formed was preferred in the second round of initiations over a second template added at the same time as nucleotides. Because only a small percentage of the promoters present during the first incubation were actually transcribed, we could not show conclusively that the same promoters were transcribed in the first and second round of initiations. However, that is the simplest explanation for the observation that the first template was preferred to the second for the following reason. A model that proposed use of different DNA1 promoters in preference to DNA2 promoters in the second round of initiations would have to include an explanation for why these DNA1 promoters became activated only after transcription from other DNA1 promoters. We showed in the experiment of Fig. 6 that the extent of rapid start complex formation was not determined by the amount of a limiting transcription factor. Therefore, the simple explanation that release of transcription factors from elongation complexes was necessary before reinitiation could occur is unlikely, although not ruled out completely. However, if the release of a factor or factors were necessary for reinitiation,
then we have narrowed the possibilities for when this release can occur. We know from experiments in the fractionated system that reinitiations from the major late promoter required the presence of all four nucleoside triphosphates. Omission of GTP, which resulted in elongation only to +10, did not allow reinitiation (1). On the other hand, experiments in which the elongation rate was altered by changing the concentration of UTP showed that reinitiation did not depend on completion of transcripts. These considerations together suggest that the promoters themselves were the components that were reused during reinitiation.

We do not yet know whether the factor(s) involved in committing the template to transcription in the second round are the same as those involved in the template commitment step defined in the earlier paper (1). Indeed, as the previous work used a transcription system reconstituted with fractions, it is possible that not all the factors involved in transcribing the major late promoter in the nuclear extract were present. For example, the partially purified system probably did not contain a significant amount of the transcription factor USF, which has been shown recently by several groups to bind to a sequence upstream of the TATA box in the major late promoter and to increase the efficiency of transcription of this promoter in vitro (5–7). The majority of USF in the nuclear extract has been found to fractionate in the P11 0.3 M step fraction (9), which was not included in our reconstituted system. We do not yet know whether USF is involved in the continued commitment of the major late promoter beyond the first round of initiations nor is it possible from the data presented here to determine whether the step that is circumvented in the second round of initiations was rate-limiting in the first round. These mechanistic details will be pursued with further analysis.

Our finding that transcription remained committed to a template containing the major late promoter for more than one round of initiations is in contradiction with the published results of Safer et al. (19) who failed to find such a preference for the first template in a transcription system reconstituted in vitro with whole cell extract. Apart from the obvious possible differences between the whole cell and nuclear extracts, another potential explanation for the difference between their results and those reported here is the number of rounds of transcription initiation that were observed in each case. In our experiments, the equivalent of only two rounds of transcription were completed. In the reactions of Safer et al. (19) the implication was that more than two rounds of transcription were observed, because they reported equal transcription from the template present during the initial incubation and the template added subsequently. In two rounds of transcription, one would expect to observe a greater level of transcript from the first template, to which the first round would be committed. Because Safer et al. (19) did not report either the actual number of transcripts produced in the multiple-round experiment or the number of transcripts expected in one round of initiations, it is not possible to resolve unambiguously the discrepancy between the two observations.

Our model for the events occurring during reinitiation of transcription from the major late promoter in vitro is consistent with the recent observation in vivo of Mattaj et al. (20) who found that the promoter for the Xenopus U2 gene was transcribed for multiple rounds when injected into Xenopus oocytes, whereas a second template containing the U2 promoter, when injected after the first template, was not transcribed. The structure of the promoters for the small nuclear RNA genes does not bear an obvious resemblance to that of other class II promoters, including the major late promoter (21). However, it is interesting to speculate that the stable sequestration of some transcription factors plays a role in regulation of class II genes. The implications of such a mechanism for gene regulation have been discussed by others (22).

Properties of the Nuclear Extract System—This study revealed three properties of the nuclear extract system that have important implications both in suggesting better ways to design reaction protocols and in advising caution in the interpretation of results obtained with this system. First, we found that nucleoside triphosphates were very rapidly degraded in the nuclear extract and, more important, that this degradation had a marked effect on the ability of transcription complexes at the major late promoter to initiate. This result suggested that an assay that measured the amount of transcript produced in one round of initiations from preformed rapid start complexes would be more reproducible and possibly also more efficient than an assay that measured transcript accumulation in a 30- or 60-min incubation at 30 °C following the initial mixing of all required transcription components. Both of these predictions were borne out by the experiment of Table I.

A second property of the nuclear extract system that has practical importance is related to the nucleotide requirements for productive initiation. We showed previously that addition of both ATP and CTP was required for productive initiation from the major late promoter in a fractionated system free of endogenous nucleoside triphosphates. In contrast, we found that with the nuclear extract system, addition of any single nucleoside triphosphate to a concentration of 600 μM was sufficient to allow some productive initiation to occur if phosphocreatine was also present. A lower concentration of ATP (100 μM) also promoted a detectable level of productively initiated transcription in the presence of phosphocreatine. It is likely that this result reflected the presence of pools of nucleoside diphosphates and/or monophosphates as well as the enzymes required to transfer phosphate groups, and that addition of any nucleoside triphosphate to the extract resulted in an increased concentration of all four nucleoside triphosphates. A similar phenomenon has been described previously for other cell extracts (23–25).

The third finding of practical significance was that the extent of formation of rapid start complexes was not limited by the availability of transcription factors. Therefore, the number of active transcription complexes formed in these extracts cannot be used reliably as a basis for calculations of the amounts of transcription factors in extracts or comparisons of the amount of factors in different types of extracts.

REFERENCES

1. Hawley, D. K., and Roeder, R. G. (1985) J. Biol. Chem. 260, 8163–8172
2. Matsui, T., Segall, J., Weil, P. A., and Roeder, R. G. (1980) J. Biol. Chem. 255, 11992–11996
3. Samuelis, M., Fire, A., and Sharp, P. A. (1982) J. Biol. Chem. 257, 14149–14157
4. Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983) Methods Enzymol. 101, 582–598
5. Sawadogo, M., and Roeder, R. G. (1985) Cell 43, 165–175
6. Carthew, R. W., Chodosh, L. A., and Sharp, P. A. (1985) Cell 43, 409–445
Supplementary Material to
Proteinase K digestion of adenovirus DNA.
Diane X. Helyar and Robert J. Roeder

Experimental Procedures

Materials—Nucleotides (Pharmacia, Kalamazoo, MI), ethidium bromide, trypsin, protease, and colcemid were purchased from Sigma. Restriction enzymes were purchased from New England BioLabs. Tris-HCl was purchased from BDH. MgCl2 was purchased from Calbiochem. PGE1, PGE2, and PGE3 were purchased from Research Biochemicals. 

Nucleotide and DNA templates—Nucleic acid extracts were prepared as described previously [17], except that all buffers contained 20 mM Tris-HCl, pH 7.5 at 4°C instead of Tris buffer. The extracts were stored at -20°C in excess. The DNA was precipitated with ethanol at 4°C, 95% ethanol, and 0.3 M NaAc, pH 4.5.

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Probes for hybridization—The specific probes were the same as used in previous experiments [17]. The probes were labeled with 32P by the random priming method [18].

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tested the effect of 5-BrU on the rate of 6TP degradation in the absence of phosphoribosyltransferase, and found that 0.02% 5-BrU had no effect, whereas 0.2% 5-BrU increased the half-life of 6TP to about 10 min. We did not determine whether 5-BrU affected the degradation rate in the presence of phosphoribosyltransferase.

We also analyzed the effect of 5-BrU on the rate of transcription when the reaction mixture was incubated with phosphoribosyltransferase. We found that the half-life of 5-BrU increased from 3 min to 15 min after 60 min incubation with phosphoribosyltransferase. These results suggested that 5-BrU was not toxic to the transcriptional system within the incubation time normally used.

To determine whether the positive effects of 5-BrU on the rate of transcription were due to its ability to inhibit the degradation of 6TP, we performed experiments with 5-BrU alone and with 5-BrU plus 6TP. We found that the half-life of 6TP increased from 3 min to 15 min after 60 min incubation with phosphoribosyltransferase. These results suggested that 5-BrU was not toxic to the transcriptional system within the incubation time normally used.

A comparison of the results obtained with 5-BrU and with 6TP is shown in Table 1. The data indicate that 5-BrU is a much more effective inhibitor of 6TP degradation than 6TP. However, the effects of 5-BrU and 6TP are not additive. When the transcription reaction was incubated with both 5-BrU and 6TP, the half-life of 6TP was increased to 15 min, but the rate of transcription was not significantly increased.

Table 1: Comparison of the half-life of 6TP in the presence and absence of phosphoribosyltransferase.

| Extract | 5-BrU | 6TP | 5-BrU + 6TP |
|---------|-------|-----|-------------|
| 0.02%   | 3.15  | 0.25| 0.02% 5-BrU |
| 0.2%    | 3.15  | 0.25| 0.1% 5-BrU  |

These results suggested that 5-BrU was not toxic to the transcriptional system within the incubation time normally used.