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Isolation of stable preinitiation, initiation, and elongation complexes from RNA polymerase II-directed transcription

(adenovirus/promoter/HeLa extract/run-off transcript/transcription initiation factor)

BRIAN SAFER, LINDA YANG, H. ESER TOLUNAY, AND W. FRENCH ANDERSON

Section on RNA and Protein Biosynthesis, Laboratory of Molecular Hematology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205

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ABSTRACT **Distinct RNA polymerase II transcription preinitiation, initiation, and elongation complexes can be formed *in vitro* on cloned adenovirus 2 DNA sequences containing the major late promoter. These transcription complexes are stable and can be rapidly isolated by gel filtration of HeLa whole cell extracts. In the absence of exogenous nucleotides and under appropriate salt conditions, a stable but transcriptionally incomplete preinitiation complex is formed. When this complex is incubated in the presence of adenosine or deoxyadenosine triphosphates, the β–γ phosphodiester bond is hydrolyzed, and RNA polymerase II joins the complex, thereby converting it into a stable initiation complex capable of forming (but prior to the formation of) the first phosphodiester bond. When this complex is isolated and incubated in the presence of all four nucleoside triphosphates, it is converted into an elongation complex that then permits the synthesis of phosphodiester bonds and the correct run-off transcript. A limiting transcription component is sequestered in the preinitiation complex. This factor is released upon elongation and can reassociate with new DNA templates during subsequent rounds of initiation. Therefore, class II genes do not appear to form activated transcription units stable for multiple rounds of transcription; rather, their transcriptional activity may be controlled in part by regulating the association of transcription factors at each initiation event.**

Correct initiation by RNA polymerase II (RNA Pol II) and elongation to generate the run-off transcript specified by truncated adenovirus type 2 (Ad2) major late promoter (MLP) DNA templates requires at least three transcription initiation factors (1–5). In conjunction with specific recognition sequences, it has been hypothesized that these factors bind to the DNA template and promote the specific binding of RNA Pol II (6–13). In addition to these general factors required for all class II genes, specific factors required for recognition of viral promoters have been identified (14). The general method used for purification of these factors has been to fractionate active cell-free extracts by conventional chromatographic procedures. Factors have been identified as those column fractions that must be combined to restore transcriptional activity. While such an approach has produced significant purification of factors, the low transcriptional activity of most cell-free extracts and further losses of activity with increasing purification have hindered identification and characterization of the components responsible for transcriptional activity.

An alternative approach would be to interrupt the transcription process at specific stages of initiation and elongation. Then, distinct preinitiation, initiation, and elongation complexes would be isolated, and their composition could be analyzed and compared. The identification of polypeptides, different from those of RNA Pol II, in these intermediate complexes would suggest their participation in the assembly of active transcription initiation complexes. We have taken the first step in this process by establishing conditions whereby preinitiation, initiation, and elongation complexes can be isolated and characterized.

MATERIALS AND METHODS

Transcription Complex Formation. HeLa whole-cell extract (WCE) prepared by the method of Manley *et al.* (15) was obtained from Bethesda Research Laboratories. Incubation mixtures (25 μl) contained 12 μl of HeLa WCE dialyzed against buffer A [20 mM Tris·HCl, pH 7.9/40 mM $(NH_4)_2SO_4$/6 mM $MgCl_2$/0.2 mM EDTA/2 mM dithiothreitol/15% glycerol], 0.4 units of creatine phosphokinase, and final concentrations of 40 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, and 10 mM creatine phosphate. The final concentration of DNA template was 60 μg/ml. For formation of stable transcription initiation complexes, incubations were supplemented with 100 μM ATP or dATP. Elongation of initiation complexes to generate the correct run-off transcript was started by the addition of 100 μM ATP, UTP, and CTP; 10 μM GTP; and 10 μCi (1 Ci = 37 GBq) of [α-^{32}P]GTP (Amersham; 400 Ci/mmol). Reinitiation of transcription after run-off could be prevented by increasing the $(NH_4)_2SO_4$ concentration to 100 mM and adding 1.7 mM $MnCl_2$ prior to elongation (16). Reaction mixtures were incubated at 25°C for the indicated period of time. Analysis of transcription products was as published (17) except that reactions were terminated by adding 1.5 vol of 0.1 M Tris·HCl (pH 6.5) containing 2% $NaDodSO_4$ and 0.4% bromophenol blue, followed by heating at 60°C for 10 min. Samples were electrophoresed on 8% acrylamide gels in TBE buffer (100 mM Tris borate/2 mM EDTA, pH 8.3). Autoradiograms of the wet gels were obtained at room temperature with Kodak AR x-ray film and DuPont Cronex Lightning-Plus intensifying screens. The correct size of the transcription products was confirmed previously (16).

Sepharose CL-2B Chromatography. Incubations (200–400 μl) were chromatographed on a 0.9 × 40 cm Sepharose CL-2B column equilibrated with buffer A. This allowed the transcriptional activity of each fraction to be evaluated directly under standard assay conditions. The flow rate was 10 ml/hr. To evaluate the polypeptide composition of column fractions, aliquots were precipitated with 2 vol of acetone and centrifuged at 8000 × g for 10 min. The pellets were dried at room temperature and brought up in 20–40 μl of sample buffer (0.5 M Tris·HCl, pH 6.5/2% $NaDodSO_4$/2 mM dithiothreitol). Samples were electrophoresed on 0.75-mm-thick Laemmli $NaDodSO_4$ gels as published (17). Polypeptides

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Abbreviations: WCE, whole-cell extract; Ad2, adenovirus type 2; MLP, major late promoter; nt, nucleotide; RNA Pol II, RNA polymerase II; kb, kilobase.

were stained with 0.2% Coomassie blue in 7.5% acetic acid/50% methanol. The distribution of DNA was determined after staining by ethidium bromide following a 5-min incubation of the gels in 0.15 M Tris·HCl buffer (pH 8.5) or, alternatively, by autoradiography of [^{32}P]-labeled template as described (17).

Templates. Two plasmids containing the Ad2 MLP were used. pSmaF contained the 2.4-kilobase (kb) *Sma* I Ad2 fragment cloned into the unique *Sma* I site of pBR313 (16). pUC8Ad2 contained the 0.8-kg *Stu* I/*Sma* I fragment of Ad2 cloned into the unique *Sma* I site of pUC8. The size of the specific run-off transcript of either plasmid truncated with *Sma* I or *Hinc*II was 536 and 405 nucleotides (nt), respectively. Since large DNA templates tended to precipitate under transcription assay conditions, DNA containing the Ad2 MLP was always transcribed after a double restriction endonuclease digestion to release the Ad2 sequences from plasmid DNA. Ad2 MLP_{405} is the designation of plasmid pUC8Ad2 digested with *Xho* I/*Hinc*II to produce a 405-nt run-off transcript; Ad2 MLP_{536} is the designation of pUC 8Ad2 digested with *Xho* I/*Pst* I to produce a 536-nt run-off transcript.

RESULTS

Distinct RNA Pol II Initiation and Elongation Complexes Can Be Formed *in Vitro*. Under standard conditions used to transcribe class II genes, the correct 536-nt transcript of Ad2 MLP_{536} was formed at a linear rate after an initial lag of ≈5 min (Fig. 1, combined conditions AB). Since the rate of elongation (≈10 nt per sec) (18) should allow formation of the correct 536-nt transcript within 1–2 min, the formation of new initiation complexes, their conversion to elongation complexes, and the termination of RNA products by run-off transcription should occur throughout the 30-min incubation period. Only 1–3 RNA transcripts per 100 DNA templates are produced in these systems (15, 16, 19). It is not possible, therefore, to determine unambiguously whether such transcriptional activity represents recycling of a small subset of active transcriptional components or a random recruitment of components required for initiation.

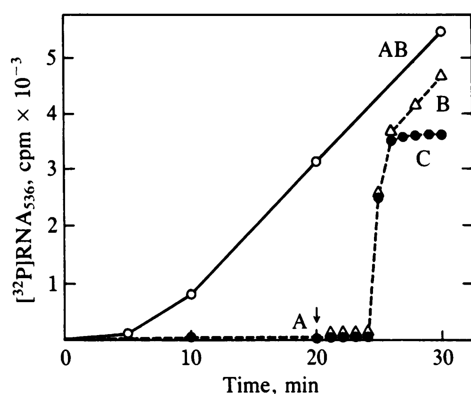

FIG. 1. Initiation and elongation of Ad2 MLP_{536} transcripts in HeLa WCE. The rate of incorporation of [^{32}P] into the correct 536-nt run-off transcript under the following incubation conditions (A, A then B, A then C, or AB combined) is presented. Under condition A, 12 μl of HeLa WCE (96 μg of protein) was incubated in a total of 25 μl containing 12 mM Tris·HCl (pH 7.9), 40 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 10 mM creatine phosphate, 0.4 units of creatine phosphokinase, 9% glycerol, 100 μM ATP or dATP, 40 μCi of α-[^{32}P]GTP per ml, and 60 μg of pSmaF digested with *Sma* I and *Xho* I (Ad2 MLP_{536}) per ml. For condition B, ATP, UTP, and CTP (100 μm each) and GTP (10 μM) were added to condition A (indicated by arrow) after 20 min of incubation (conditions A then B). For condition C, all four nucleoside triphosphates, $(NH_4)_2SO_4$ (final concentration, 100 mM), and 1.7 mM $MnCl_2$ were added to condition A after 20 min of incubation (conditions A then C). For combined incubation conditions AB, components of conditions A and B were present at the start of incubation.

Conversion of initiation to elongation complexes can be interrupted, however, by omitting GTP, UTP, and CTP from the transcription system. Assembly of initiation complexes is dependent on either ATP or dATP having hydrolyzable β–γ phosphodiester bonds (17, 20, 21). Because it is difficult to totally remove endogenous ribonucleoside triphosphates from HeLa WCE (22, 23), it is advantageous to use dATP that can support initiation efficiently but cannot be incorporated into RNA. After 20 min of incubation on the absence of exogenous ATP, UTP, CTP, and GTP (Fig. 1, condition A), the correct 536-nt run-off transcript was generated rapidly upon provision of all four ribonucleoside triphosphates. When the $(NH_4)_2SO_4$ concentration was increased simultaneously to 100 mM and 1.7 mM $MnCl_2$ was added, reinitiation was inhibited and only elongation of preformed initiation complexes occurred (Fig. 1, condition A and then C). When the ionic strength was not increased, reinitiation could occur after the addition of nucleoside triphosphates (Fig. 1, condition A and then B). Transcription initiation complexes did not continue to accumulate beyond 20 min of incubation under condition A (data not shown). Therefore, their formation appears to be limited by the availability of a component other than RNA Pol II or the Ad2 MLP template, both of which were present in large excess (see below). In addition, the data show that the rate-limiting step of *in vitro* transcription occurs at initiation.

Stability of Transcription Initiation Factor Binding in Initiation Complexes. When two DNA templates with equal transcriptional efficiency are added to HeLa WCE, the relative extent of their transcription is determined by their order of addition (3). Transcription of two truncated Ad2 MLP templates having run-off RNA products of 536 and 405 nt (Ad2 MLP_{536} and Ad2 MLP_{405}) is shown in Fig. 2. Ad2 MLP_{536}, specifying the 536-nt run-off transcript, was added to HeLa WCE under conditions optimal for initiation complex formation (Fig. 2*A*). The second DNA template, Ad2 MLP_{405}, was omitted, added simultaneously, or added after incubation for 2, 4, 6, 8, or 10 min at 25°C. After 10 min of further incubation,

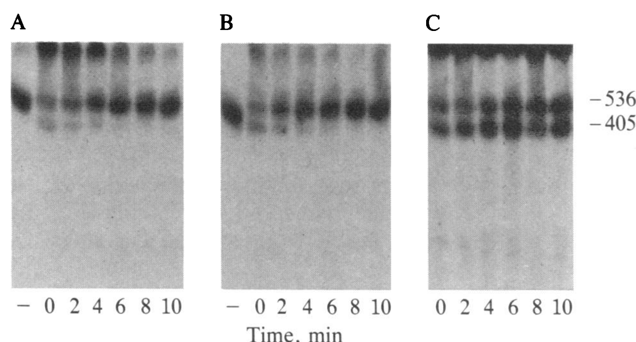

FIG. 2. Sequestration of transcription initiation factor(s) during preinitiation complex assembly. HeLa WCE was incubated under ionic conditions optimal for initiation complex formation with 40 μg of Ad2 MLP_{536} per ml truncated with *Sma* I (536-nt run-off transcript) for the indicated period of time. Only the incubation in *A* contained 100 μm dATP. An equal amount of Ad2 MLP_{405} truncated with *Hinc*II (405-nt run-off transcript) was then added at the indicated times, and the incubation was continued for an additional 10 min. The final total DNA concentration was 80 μg/ml. Individual reactions were then supplemented with all four nucleoside triphosphates either in the presence (*A* and *B*) of additional $(NH_4)_2SO_4$ (final concentration, 100 mM) and $MnCl_2$ (1.7 mM) to prevent reinitiation or in the absence of these additions (*C*) to allow reinitiation. After incubation, the run-off transcription products were analyzed by autoradiography after PAGE on 8% gels. Exposure time for *C* was less than that for *A* and *B*.

the assembled initiation complexes were elongated under conditions that prevented reinitiation. The synchronous addition of both templates at 0 min resulted in approximately equal transcription of both DNA templates. With increasing times of incubation with the first template alone, there was a progressive loss in the ability of the extract to transcribe the second template. By 8 min of incubation, the ability to transcribe the second template was lost completely, while the first DNA template was transcribed as if it alone were present. Continued incubation with the second template for periods up to 60 min prior to shifting to elongation conditions did not alter these results (data not shown). The data suggest that, with increasing time of incubation, a limiting transcriptional component is sequestered by the first template and is no longer available when the second template is added. Sequestration of a transcriptional component by the first template was also obtained if the order of template addition was reversed (data not shown).

Although initiation complex formation requires an adenine nucleoside triphosphate having a hydrolyzable β–γ phosphodiester bond, binding of the limiting component by the first-added DNA template does not. This conclusion is based on the observation that identical results were obtained in the absence of dATP (or ATP) (Fig. 2*B*). Sequestration of the limiting factor in the absence of dATP (or ATP) was maintained for as long as 60 min (data not shown).

In contrast to the results shown in Fig. 2 *A* and *B*, transcription of the second template occurred efficiently when elongation was allowed to proceed under conditions that allow reinitiation (Fig. 2*C*). It appears, therefore, that the limiting factor is released from the first template upon elongation and subsequently can reassociate randomly with either the first or second DNA template if reinitiation is allowed to occur.

Isolation of Transcription Complexes by Sepharose CL-2B Chromatography. Before conversion to elongation complexes, initiation complexes are sensitive to the ionic detergent Sarkosyl (17, 24). Once elongation has started, Sarkosyl does not inhibit formation of the run-off transcript. Transcription complexes formed on Ad2 MLP in dATP-supplemented HeLa WCE are stable Sarkosyl-sensitive initiation complexes that can be isolated by glycerol gradient centrifugation (17). To identify the polypeptide components required for their assembly, a preparative procedure having greater resolution was required.

HeLa WCE incubated with an Ad2 MLP template in the presence of dATP was chromatographed on Sepharose CL-2B. This procedure resulted in the extensive purification of stable and active transcription initiation complexes (Fig. 3). The A_{254} profile of the Sepharose CL-2B column (Fig. 3*A*) showed a sharp peak at the void volume (V_0) that was well-separated from most HeLa WCE components, which were eluted in fractions 40–80. This coincided with the distribution of the Ad2 MLP template DNA (data not shown). When column fractions were assayed in the presence and absence of α-amanitin at 1 μg/ml, the distribution of RNA Pol II was shown to be bimodal. (Fig. 3*B*). When Ad2 MLP initiation complexes were formed, ≈20% of RNA Pol II activity was eluted at V_0. In the absence of the Ad2 MLP template, RNA Pol II was found only in fractions 40–80 (data not shown).

Active transcription initiation complexes could be detected by supplementing column fractions with α-[^{32}P]GTP and all four nucleoside triphosphates. The specific 536-nt run-off transcript was only produced by fractions that were eluted at V_0 (Fig. 3*C*). Identical results were obtained when elongation was performed either under conditions of low ionic strength established for coupled initiation and elongation or by a single round of elongation at high ionic strength (data not shown). When column fractions also were assayed and supplemented

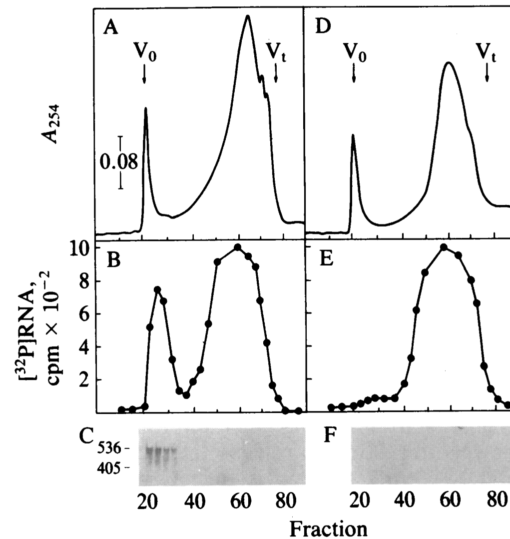

FIG. 3. Transcription complex isolation by Sepharose CL-2B chromatography. Ad2 MLP_{536} and HeLa WCE incubated under condition A (see Fig. 1) were chromatographed on a 0.9 × 40 cm Sepharose CL-2B column equilibrated with buffer A. The column eluate was monitored at A_{254} (—) (*A*) and individual 500-μl fractions were assayed for RNA Pol II activity (•) by the method of Hodo and Blatti (25) (*B*). In *C*, individual column fractions were supplemented with all four nucleoside triphosphates and α-[^{32}P]GTP, followed by incubation for 20 min; only fractions in the void volume generate the correct 536-nt run-off transcript. A duplicate assay in which all fractions also were supplemented with Ad2 MLP_{405} gave exactly the same pattern as shown in *C*. An identical incubation and analysis are presented in *D–F*, with the exception that dATP and creatine phosphate were omitted from the initial incubation of Ad2 MLP_{536} with HeLa WCE.

with a second Ad2 MLP template truncated to produce a 405-nt run-off transcript, the 405-nt transcript was not produced (i.e., the same pattern as seen in *C* was obtained). Assembly of specific transcription complexes capable of correctly transcribing Ad2 MLP also was not produced by incubation of HeLa WCE with only the pBR313 moiety of the pSmaF constructs or in the absence of DNA template.

To estimate the purification achieved by gel filtration, the polypeptide composition of every fifth fraction was analyzed by $NaDodSO_4$/PAGE (Fig. 4). Considerable purification of the transcription initiation complex was achieved by gel filtration. As estimated by densitometry of the Coomassie blue-stained gel, $<1 \times 10^{-3}$ of the proteins in the HeLa WCE were found in the V_0 fraction containing the initiation complex. Distinct polypeptide patterns coincident with the transcription complexes were characteristic of each functional state and will be reported elsewhere.

Therefore, it appears that transcription initiation complex formation requires an assembly of components that recognize specific promoter sequences on the Ad2 MLP template. No evidence for an independent polypeptide complex having transcriptional activity was seen. In addition, not all transcription components required for formation of the transcription initiation complex were present in, or were recycled from, the isolated complex (see below). Therefore, reinitiation on a second DNA template cannot occur.

To determine if binding of RNA Pol II could account for the functional sequestration of transcriptional activity by the first-added template, HeLa WCE was incubated with Ad2 MLP_{536} template in the absence of dATP (or ATP) and then was chromatographed on Sepharose CL-2B (Fig. 3*D*). An A_{254} profile similar to that of Fig. 3*A* was obtained, and the DNA template was eluted coincident with the V_0 peak. In contrast to the results obtained by filtration of the Ad2 MLP

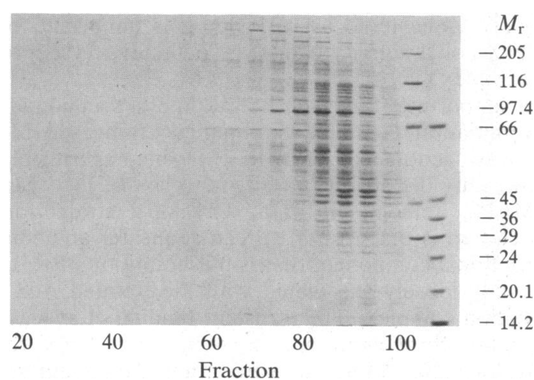

FIG. 4. Polypeptide composition analysis of the RNA Pol II transcription initiation complex obtained by Sepharose CL-2B chromatography. HeLa WCE (400 μl) was incubated and fractionated as described in the legend to Fig. 3. Proteins in 200-μl aliquots of each 400-μl column fraction were collected by precipitation with 2 vol of acetone and were analyzed by PAGE under denaturing conditions. Gels were stained with Coomassie blue. The polypeptide composition of every fifth fraction is shown. The distribution of transcriptional and other polypeptide components for the column run was essentially identical to the results shown in Fig. 3. Calibration of the gel was with known protein standards.

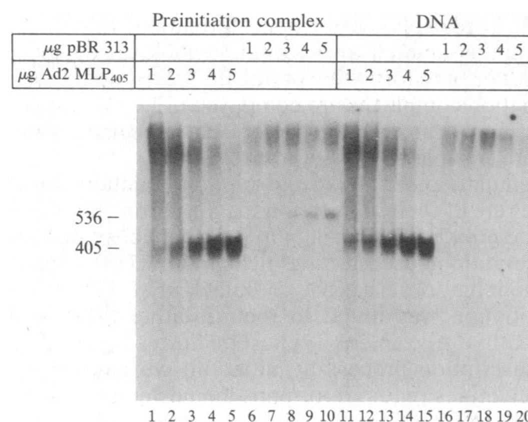

FIG. 5. Transcriptional activity of the Ad2 MLP_{536} preinitiation complex. The ability of Ad2 MLP_{536} to be transcribed in the template competition assay (see Fig. 2) was evaluated before and after incubation and isolation as described in Fig. 3 *D–F*. HeLa WCE was first incubated under condition A (Fig. 1) for 15 min in the presence of either pBR313 (40, 80, 120, 160, or 200 μg/ml) or equivalent concentrations of Ad2 MLP_{405} template. Ad2 MLP_{536} DNA (0.2 μg), either as a preinitiation complex (lanes 1–10) or free (lanes 11–20), was then added, and the incubation was continued for an additional 15 min. A single round of elongation was then performed under condition C (Fig. 1).

initiation complex, however, RNA Pol II activity was not found in stable association with the template incubated in the absence of dATP (or ATP) (Fig. 3*E*), and transcriptional activity was not detected (Fig. 3*F*). Therefore, stable binding of RNA Pol II appears to require the presence of dATP (or ATP).

Evidence for Distinct Preinitiation Complexes. Prior to binding of RNA Pol II, however, the DNA template does bind one or more transcriptional components in a stable preinitiation complex. The effect of this association on transcriptional activity of the DNA template is shown in Fig. 5. The transcriptional activity of Ad2 MLP_{536} DNA isolated as a preinitiation complex (Fig. 5, lanes 1–10) or as an equivalent amount of free DNA (Fig. 5, lanes 11–20) was compared. Since transcription in HeLa WCE appears to require the presence of nonspecific "carrier" DNA and the amount of template provided as a preinitiation complex or free DNA is limited by the concentration of the DNA in the V_0 fraction (0.2 μg/ml), the transcriptional activity of the Ad2 MLP_{536} template was compared over a 5-fold range of carrier DNA concentration added to HeLa WCE 10 min earlier. Carrier DNA was provided either as Ad2 MLP_{405} (Fig. 5, lanes 1–5 and 11–15) or pBR313 linearized with *Sma* I (Fig. 5, lanes 6–10 and 16–20).

When pBR313 was used as the carrier DNA in the HeLa WCE (final concentration from 40 to 200 μg/ml), the specific 536-nt run-off transcript was produced from the isolated preinitiation complex (Fig. 5, lanes 6–10); no transcriptional activity was seen, however, at an equivalent concentration of free Ad2 MLP_{536} DNA (Fig. 5, lanes 16–20). These results suggest that pBR313 DNA, when incubated with HeLa WCE prior to the addition of Ad2 MLP_{536}, sequesters some transcriptional component required when a competent DNA template is subsequently provided. The same component sequestered by pBR313 was stably associated in the Ad2 MLP_{536} preinitiation complex, however, so that transcriptional activity of the isolated Ad2 MLP_{536} preinitiation complex was observed over the entire range of carrier DNA concentration. When Ad2 MLP_{405} was used instead of pBR313 in this experiment, transcription of the preinitiation complex (Fig. 5, lanes 1–5) or free Ad2 MLP_{536} (Fig. 5, lanes 11–15) did not occur, and only transcription of the "carrier" DNA was seen (405-nt run-off transcript). These results suggest, therefore, that the preinitiation complex obtained by gel filtration still requires a second additional transcriptional component prior to the binding of RNA Pol II. When pBR313 was used as the carrier DNA, sequestration of the second factor did not occur and transcription of the preinitiation complex was permitted. When the Ad2 MLP was contained in the carrier DNA (i.e., as Ad2 MLP_{405}), stable binding of this additional factor occurred, and it was unavailable to the preinitiation complex. The data suggest, therefore, that at least two functionally distinct factors are present in preinitiation complexes prior to the ATP-dependent assembly of competent initiation complexes containing RNA Pol II.

DISCUSSION

We were able to form and isolate distinct preinitiation, initiation, and elongation transcription complexes. Functional evidence indicates that correct transcription of class II genes by RNA Pol II appears to require at least two additional factors for accurate initiation at the cap site. Although the functions of these factors are not known, they appear to bind to specific Ad2 MLP sequences prior to binding of RNA Pol II.

Davison *et al.* (3) first reported that the DNA template sequestered a limiting transcriptional component required for accurate initiation of RNA Pol II transcription when incubated with HeLa WCE. This sequestration prevented transcription of a second DNA template when subsequently added to the system. This suggested to us that the process of transcription initiation might be analogous to eukaryotic protein synthesis initiation, where translational initiation factors stably bind to template (mRNA) and initiating ribosomes to form an ordered sequential series of preinitiation complexes [reviewed by Jagus *et al.* (26)]. Therefore, the approach we devised to identify and purify transcription factors was to interrupt the normal transcription initiation sequence at different points to identify transcription components present at each of the intermediary stages of transcription complex formation. The method used to accumulate specific intermediary complexes was simply to omit those components required for the conversion of one complex to the next stage of transcription complex assembly. In the discussion that follows, we define elongation complexes as DNA templates containing stably bound RNA Pol II already

engaged in phosphodiester bond formation. Initiation complexes are assembled transcription complexes competent to form, but prior to formation of, the first phosphodiester bond. Preinitiation complexes are composed of DNA template and one or more transcription factors but are incomplete and, therefore, unable to initiate transcription.

Accumulation of stable transcription initiation complexes and the rate of their conversion to elongation complexes can be modulated by the availability of ribonucleotides and the ionic conditions used during incubation (17). In agreement with the initial observations of Bunick *et al.* (20), transcription initiation was found to require either ATP or dATP having a hydrolyzable β–γ phosphodiester bond. To arrest the transcription process at initiation, we omitted all four ribonucleotides required for phosphodiester bond formation and used dATP, which is not utilized as a substrate by RNA Pol II. In the absence of elongation, the rate of formation of initiation complexes for 20 min under these conditions appears to equal that found when initiation and elongation are allowed to proceed simultaneously. This observation is demonstrated in Fig. 1, where elongation of preformed initiation complexes [reinitiation prevented by high $(NH_4)_2SO_4$ and $MnCl_2$] generates a similar amount of specific run-off transcript compared to that formed during 20 min of coupled transcription (compare the level in Fig. 1 under condition AB at 20 min with the level under condition A and then C). The formation of new transcription initiation complexes beyond 20 min appears to be prevented, however, unless elongation is allowed to occur (data not shown).

The possibility that this result was due to the sequestration of a limiting transcription component was investigated by template competition studies similar to those described by Davison *et al.* (3). When saturating levels of template (Ad2 MLP_{536}) were incubated with HeLa WCE under conditions that allowed initiation but prevented elongation, the ability of a second template (Ad2 MLP_{405}) to form an initiation complex was rapidly lost (Fig. 2). This apparent sequestration of a limiting transcription component did not require ATP or dATP in agreement with previous studies (3). However, unlike the results of Davison *et al.* (3), we demonstrated that the sequestered component could be released upon elongation and then could be reutilized in a catalytic fashion. Under conditions that allowed reinitiation, the correct run-off transcripts specified by both DNA templates were produced. One possible explanation for the reversible sequestration seen in our studies is that both Ad2 MLP templates contain identical promoter sequences and are transcribed with equal efficiency. In the study of Davison *et al.* (3), two different genes (the first added in large excess) were transcribed. In addition, their HeLa WCE was supplemented with poly(I)·poly(C), which may compete with the second-added template for binding of the limiting component. Therefore, our results demonstrate that one or more transcription factors can be used in a catalytic fashion during transcription complex assembly.

Evidence for preinitiation complexes that precede the stable binding of RNA Pol II was obtained in template competition experiments by examining the transcriptional activity of DNA before and after incubation with HeLa WCE in the absence of adenosine triphosphates. The transcriptional activity of a preinitiation complex containing Ad2 MLP_{536} (resolved from most HeLa WCE components by Sepharose CL-2B chromatography) is maintained when it is added to a transcription assay to which pBR313 had previously been added (Fig. 5*A*). No transcriptional activity of an equivalent amount of untreated Ad2 MLP template is seen (Fig. 5). These results imply that Ad2 MLP stably binds a transcription component such that it remains partially activated in HeLa WCE from which the factor had been previously sequestered by pBR313. It is important to note that RNA Pol II activity cannot be detected in this preinitiation complex.

Data supporting stable sequestration of a second factor in the preinitiation complex is obtained from Ad2 MLP_{405} template is used instead of pBR313 in this experiment (Fig. 5). Now, only the 405-nt run-off transcript of the "carrier" Ad2 MLP_{405} template is seen. The most straightforward interpretation is that Ad2 MLP can sequester an additional transcriptional component that is not bound by pBR313 and that is only loosely associated with the isolated Ad2 MLP preinitiation complex. The transient binding of at least two transcription components appears to indicate, therefore, that class II genes do not form activated transcription units stable for multiple initiation events, in contrast to the stable binding of factors to class I and III genes (27–31). This mechanism of transcription initiation may allow gene expression to be regulated with the needs of the cell in a more rapidly responsive manner.

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