Promoter-dependent Transcription by RNA Polymerase II Using Immobilized Enzyme Complexes*

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DNA fragments containing the adenovirus 2 major late or simian virus 40 early promoters were attached to a solid support via a biotin-streptavidin linkage at one end of the fragment, upstream of the RNA start site. Templates immobilized in this manner were incubated with HeLa cell nuclear extracts to form preinitiation complexes containing RNA polymerase II and accessory proteins required for faithful in vitro transcription. These complexes did not require ATP or dATP for assembly, were sensitive to 0.25% Sarkosyl, and were stable to extensive washing. Their incubation with specific combinations of nucleoside triphosphates resulted in the initiation of RNA chain polymerization in situ, while addition of the remaining nucleoside triphosphates was necessary to produce a full length runoff RNA. Transcriptional activity associated with preinitiation complexes was purified approximately 300-fold, relative to the unfractionated nuclear extract. The use of immobilized template permits considerable flexibility in experimental design, as substrates and inhibitors can be added and washed out of the reaction at each step. We exploited this property of the system to dissect the temporal substrate requirements for initiation of RNA synthesis. It is known from prior work that at least one step in the promoter-dependent RNA synthesis reaction requires an adenosine nucleotide that is hydrolyzable at the β,γ-position. This requirement is independent of the initiating nucleotide and can be satisfied by dATP, which is not ordinarily incorporated into the RNA product. We show here that the β,γ-hydrolyzable adenosine nucleotide must be present simultaneously with the initiating nucleoside triphosphates. No reaction occurred when complexes were incubated with dATP, washed to remove dATP, and incubated subsequently with the two initiating nucleotides.

Faithful initiation of transcription by mammalian RNA polymerase II requires accessory transcription factors that do not co-purify with the RNA polymerase (Weil et al., 1979). Early work showed that multiple fractions, each containing one or more transcription factors, are required in addition to the RNA polymerase to reconstitute promoter-specific transcription with the adenovirus 2 major late promoter (Matsui et al., 1980). Additional, promoter-specific transcription factors are required to reconstitute activity with other promoters, such as the SV40 early promoter (Dynan and Tjian, 1985).

Several kinetic and order of addition studies have shown that the components required for initiation of transcription assemble into a promoter-bound complex prior to RNA synthesis (Davison et al., 1983; Fire et al., 1984; Hawley and Roeder, 1985). Attempts to physically isolate these complexes have met with mixed success, however. Incubation of promoter-containing DNA with cell-free extracts in the absence of nucleoside triphosphates results in the formation of insoluble aggregates that can be recovered by brief centrifugation (Culotta et al., 1985). Proteins in the pellet fraction are sufficient for transcription from templates containing promoters for RNA polymerases I and III, but efficient transcription from templates containing a promoter for RNA polymerase II requires reconstitution with the supernatant fraction. Transcription complexes can also be isolated by velocity sedimentation or gel filtration (Tolunay et al., 1984; Safer et al., 1985; Luse et al., 1987; Cai and Luse, 1987a). In some cases, formation of these complexes required exogenous ATP.

In the present work, we formed preinitiation complexes using promoter-containing DNA attached to a solid support. These complexes are formed and retained on the support, whereas the bulk of the contaminating protein is removed by washing. The promoter-containing DNA is immobilized in such a way that it remains active as a template for synthesis of runoff RNA, so that the transcription reaction can be studied in situ, without eluting the proteins from the support. This avoids the need to handle small amounts of protein in solution and the accompanying losses of activity.

The immobilized template system has been exploited to examine the substrate requirements for initiation. At least one step in the promoter-dependent RNA synthesis reaction requires an adenosine nucleotide that is hydrolyzable at the β,γ-position (Bunick et al., 1982; Sawadogo and Roeder, 1984). This requirement is independent of the initiating nucleotide and can be satisfied by dATP, which is not ordinarily incorporated into the RNA product. The underlying basis for the ATP/dATP requirement is not known. These nucleotides are not required for formation of rapid start preinitiation complexes, nor for the elongation reaction once a short nascent RNA has been formed, suggesting that the requirement occurs prior to or during the formation of the initial few phosphodiester bonds (Sawadogo and Roeder, 1984). We have used the immobilized template system to show that the hydrolyzable adenosine nucleotide must be present simultaneously with the initiating nucleoside triphosphates. No reaction occurs when complexes are incubated with dATP, washed to remove dATP, and incubated subsequently with the two initiating nucleotides.

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MATERIALS AND METHODS

RESULTS

Formation of Preinitiation Complexes—Biotinylated, promoter-containing DNA fragments were coupled to streptavidin-agarose beads and tested for their ability to support transcription. DNA beads containing the Ad2MLP or SV40 EP were incubated at 30 °C for 90 min with nuclear extract to allow the formation of preinitiation complexes. Ribonucleoside triphosphates were added, and transcription reactions were carried out as described under "Materials and Methods." Promoter-specific RNA synthesis was determined by the run-off transcription assay, which measures the production of transcripts that initiate at the promoter and terminate at the end of the restriction fragment. Transcription reactions using Ad2MLP template gave a predominant runoff RNA with an apparent size of 135 nucleotides, relative to DNA size markers (Fig. 3, lane 1). The anticipated full length transcript from this template is 133 nucleotides (Fig. 1). Transcription from the SV40 EP template gave a predominant RNA of approximately 165 nucleotides (Fig. 3, lane 9); the expected full length transcript is 162 nucleotides (Fig. 1).

When the DNA beads were washed extensively after complex formation, a substantial proportion of the transcriptional activity was recovered bound to the bead. Results were similar for both Ad2MLP and SV40 EP templates (compare lanes 1 versus 5 and 9 versus 13). These stable complexes formed at 30 °C but not at 4 °C (lanes 5 versus 8 and 13 versus 16). Once formed, the complexes could be washed at 30 °C or 4 °C with equivalent recovery of activity (data not shown). Approximately 60 min was required for complex formation to reach completion (lanes 3–5 and 11–13). The complexes were sensitive to the detergent Sarkosyl at 0.25% concentration, as expected if RNA synthesis had not yet occurred (lanes 6 and 14). Transcription was completely inhibited by α-amanitin at 1 µg/ml, demonstrating that RNA synthesis was carried out by RNA polymerase II (lanes 7 and 15).

The binding of transcriptional activity to the bead was template-dependent. No products were seen when streptavidin-agarose beads lacking DNA were incubated with nuclear extract, washed, and challenged to carry out RNA synthesis in the presence of added template and nucleotides (data not shown).

Degree of Purification of Preinitiation Complexes—The amount of protein associated with washed preinitiation complexes on DNA beads was quantitated by densitometry of Coomassie Blue-stained polypeptides in SDS-polyacrylamide gels. Preinitiation complexes were formed by incubating HeLa cell nuclear extract with Ad2MLP DNA beads under standard conditions. Samples were then divided into two portions. One portion was analyzed for protein content by SDS-polyacrylamide gel electrophoresis; the other was used for RNA synthesis.

The SDS-polyacrylamide gel analysis of protein from unwashed reactions is shown in Fig. 4, lanes 1–3. Each lane contained 1/20 of the protein from a standard 50-µl reaction. Protein eluted from washed complexes is shown in lanes 4 and 5, and protein from DNA beads not incubated with extract is shown in lanes 6 and 7. Each of lanes 4–7 contained material from the equivalent of 1.5 standard reactions. Although the washed complexes contain only a small fraction of the protein from the unwashed reactions, the pattern of polypeptides remains complex, and it is likely that much of the material represents residual contaminants (see "Discussion"). A prominent band at the dye front was seen in all samples; this may represent streptavidin that has been stripped from the bead by SDS treatment.

The total amount of protein in each sample was estimated by densitometry of each entire lane. Neglecting the dye front, there was approximately 15-fold more protein in lanes 1–3 than in lanes 4 and 5. After normalization for the fraction of the sample loaded, this suggests that unwashed reactions contain 450-fold more protein than is present in the washed complexes. In this experiment, there was a 65% recovery of transcriptional activity after washing (data not shown), so that the apparent purification of transcriptional activity was approximately 300-fold.

Attempts were made to increase the degree of purification by washing the preinitiation complexes with buffers that contained KCl or the detergents Nonidet P-40, Triton X-100, or Sarkosyl at various concentrations. These treatments gave
little additional purification and were not adopted for routine use. The effect of adding poly[d(I-C)] as a carrier polynucleotide during complex formation was also tested. In the range from 0 to 1 µg per reaction, poly[d(I-C)] had little effect on the formation or purity of the bound transcription complexes (data not shown).

**Nucleotide Requirements for Initiation**—Prior studies have shown that transcription complexes that have not yet initiated RNA synthesis are sensitive to treatment with >0.1% Sarkosyl (Hawley and Roeder, 1985). Complexes become Sarkosyl-resistant after incubation with combinations of nucleoside triphosphates that allow formation of the first phosphodiester bond in the RNA (Ackerman et al., 1983; Hawley and Roeder, 1985). This progression from a Sarkosyl-sensitive to a Sarkosyl-resistant state was used to follow the initiation reaction in the DNA-bead system.

Preinitiation complexes were allowed to form on DNA beads and washed by the standard procedure. Complexes were incubated with various ribonucleoside triphosphate combinations for 5 min at 30 °C, then challenged to synthesize a runoff RNA product in the presence of 0.4% Sarkosyl and the remaining nucleoside triphosphates. Ad2MLP complexes became Sarkosyl-resistant after incubation in the presence of ATP and CTP. SV40 EP complexes became Sarkosyl-resistant after incubation in the presence of ATP and GTP (Fig. 5A, lanes 5 and 14). Other combinations of ribonucleoside triphosphates were not effective. The Ad2MLP is expected to have one major initiation site (underlined) within the sequence 5’ CTCACTCTT ... 3’ (Gelinus and Roberts, 1977; Ziff and Evans, 1978; Weil et al., 1979). The exact start site for the SV40 EP is less certain, but primer extension experiments suggest that the RNA 5’ ends map to several sites (underlined) within the sequence 5’ CGGCCCTGAGCC ... 3’ (Dyman and Tjian, 1983) thus, the nucleotide combinations that were effective in our system are consistent with utilization of the major initiation site of the Ad2MLP, and one of the probable initiation sites of the SV40 EP. The data suggest that dinucleotide synthesis was sufficient to render the complexes resistant to Sarkosyl, although they do not rule out the possibility that trace cross-contamination of the nucleoside triphosphates was sufficient for synthesis of longer oligonucleotides.

Prior work has shown that ribonucleoside triphosphate combinations lacking ATP will not support initiation, even if the two initiating nucleoside triphosphates are present (Bunick et al., 1982; Sawadogo and Roeder, 1984). This requirement for ATP can also be satisfied by dATP, even though the latter is not incorporated into the newly synthesized RNA (Sawadogo and Roeder, 1984). To obtain a more complete picture of the ribonucleoside triphosphate combination that would support initiation, the experiment shown in Fig. 5A was repeated with dATP present during the initiation reactions. Conversion to a Sarkosyl-resistant form occurred when Ad2MLP complexes were allowed to initiate with dATP, ATP, and CTP (Fig. 5B, lane 5) and also, unexpectedly, with dATP, CTP, and UTP (lane 9). The latter combination might allow initiation of RNA synthesis at the second, third, or fourth position after the normal initiation site. With SV40 EP complexes, synthesis of the correct runoff RNA occurred as expected with dATP, ATP, and GTP (lane 16) and with dATP, GTP, and CTP (lane 18). In addition, a smaller RNA of approximately 150 nucleotides was produced from the SV40 EP with dATP, GTP, and CTP (lane 18) or with dATP, CTP, and UTP (lane 19).

The nucleotide-dependent progression to Sarkosyl resistance observed in these experiments provides a clear demonstration that the complexes that are recovered initially on the washed beads have not yet begun to synthesize RNA. These
experiments also show that the requirement for β,γ-hydrolyzable adenosine nucleotide for initiation is retained in the purified DNA-bead transcription system.

**Role of Endogenous Nucleotides**—Whole cell and nuclear extracts often contain endogenous nucleotides that can interfere with studies of the transcription reaction. We wished to determine whether endogenous nucleotides were present in our nuclear extract, and, if so, whether they were effectively removed by washing the preinitiation complexes bound to the DNA beads.

Preinitiation complexes were formed by incubating HeLa cell nuclear extract with Ad2MLP DNA beads. In the experiment shown in Fig. 6, lanes 1–4, the unwashed complexes were incubated with CTP, GTP, and the β,γ-nonhydrolyzable ATP analogue, AMP-PNP. Like other nucleotide imidodiphosphates, AMP-PNP is efficiently incorporated into RNA by a nucleotidyltransferase reaction, with release of the imidodiphosphate moiety. AMP-PNP cannot fully substitute for ATP by RNA polymerase II, however, presumably because of its nonhydrolyzable β,γ-imido linkage (Bunick et al., 1982; Sadagopan and Foeder, 1984). After incubation for 5 min at 30 °C, complexes were challenged to synthesize runoff RNA in the presence of 0.4% Sarkosyl and the remaining nucleoside triphosphates. Even though no exogenous hydrolyzable adenine nucleotide was present during the initiation phase of the reaction, a small amount of RNA synthesis occurred, suggesting that some endogenous ATP or dATP was present. RNA synthesis was greatly stimulated, however, when 1–100 μM dATP was included in the initiation phase of the reaction (Fig. 6, lanes 2–4).

The same experiment was carried out using complexes that were washed before the addition of nucleotides (lanes 5–8). In this case, there was no detectable RNA synthesis in the absence of exogenous hydrolyzable adenosine nucleotide, indicating that washing the preinitiation complexes effectively removed endogenous nucleotides that were present in the nuclear extract. When these reactions included 10 μM or 100 μM dATP (lanes 7 and 8), high levels of RNA synthesis were seen.

The finding that endogenous ATP or dATP was present in the extracts raised the possibility that these nucleotides might contribute in some way to the formation of preinitiation complexes. Although this and the previous experiment showed that the ATP/dATP requirement for initiation has not been satisfied at the time of preinitiation complex formation, it is possible that these nucleotides might be required at additional, earlier steps during the promoter recognition reaction. Prior studies indicated that ATP was necessary to stabilize transcription complexes prior to isolation (Tolunay et al., 1984; Safer et al., 1985).

To further investigate the role of endogenous nucleotides, we enzymatically depleted ATP and dATP from nuclear extracts. Hexokinase and dextrose were added to nuclear extracts, and the mixture was incubated for 15 min at 30 °C. Hexokinase transfers the γ-phosphate from ATP (or dATP) to specific β,γ-cleavable acceptors to yield a phosphorylated sugar and ADP (or dADP). In the absence of an appropriate sugar acceptor, there is no reaction. The treated nuclear extract was incubated with Ad2MLP DNA beads to form preinitiation complexes. These complexes were washed to remove hexokinase and dextrose and tested for transcriptional activity by the standard method. Preincubation of the extract with hexokinase and dextrose had no effect on the final level of RNA synthesis (Fig. 7, lanes 6–8). Similar results were obtained when nuclear extracts were preincubated with apyrase, a phosphohydrolase that utilizes dATP and ATP as substrates (data not shown).

As controls, some reactions were carried out where initiating nucleotides were deliberately added to nuclear extracts during the preincubation with hexokinase and dextrose. Ad2MLP DNA beads were added, and incubation was continued to allow potential initiation of RNA synthesis. Samples were then treated with 0.25% Sarkosyl to disrupt noninitiated complexes. Finally, complexes were washed and challenged to make runoff RNA in the presence of all four nucleoside triphosphates. In these experiments, RNA synthesis was observed with untreated nuclear extracts, or when hexokinase or glucose was present individually during the preincubation. When hexokinase and dextrose were present together, however, no RNA synthesis was observed, showing that the ATP required for initiation had been depleted under the conditions used (Fig. 7, lane 4).

We conclude from these experiments that endogenous ATP and dATP are not essential to the formulation of stable preinitiation complexes at the Ad2MLP. These experiments illustrate the utility of an immobilized template system. Because the hexokinase, dextrose, and apyrase can be removed from the reaction by washing the DNA beads, their effect on preinitiation complex formation can be tested without inhibiting subsequent steps of transcription.

**β,γ-Hydrolyzable Adenosine Nucleotide Must Be Present Simultaneously with Initiating NTPs**—The previous experiments confirmed that β,γ-hydrolyzable adenosine nucleotide was required for initiation of RNA synthesis by purified preinitiation complexes. To further dissect the substrate requirements for initiation, a new reaction protocol was intro-

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Fig. 7. Effect of pretreatment with hexokinase and dextrose. HeLa cell nuclear extracts were incubated with hexokinase (0.1 unit), dextrose (4 mM), and initiating nucleotides (1 mM AMP-PNP, 1 mM CTP, and 100 μM dATP) as indicated. Incubation was continued for 15 min at 30 °C. Ad2MLP DNA beads (500 ng/reaction) were added, and incubation continued for 60 min at 30 °C. Sarkosyl concentration was adjusted to 0.25% w/v where indicated. Complexes were washed, initiation and elongation reactions were carried out, and products were analyzed as described under "Materials and Methods."

3 J. A. Arias and W. S. Dynan, unpublished data.
FIG. 8. Requirement for dATP/ATP during the initiation of transcription. HeLa cell nuclear extract was mixed with Ad2MLP DNA beads (800 ng/reaction). Incubation and washing was carried out as described under "Materials and Methods." Combinations of nucleotides were added as indicated (100 μM each), and incubation continued for 10 min at 30°C. The DNA beads were washed again 5 times with 500 μl of 0.5 × TM 0.1 at 4°C. Additional nucleotides were added as indicated (100 μM each), and incubation continued for 10 min at 30°C. The concentration of Sarkosyl was adjusted to 0.4%, and elongation reactions and analysis of products were carried out as described under "Materials and Methods.'

duced using two sets of washes. Preinitiation complexes were formed using Ad2MLP DNA beads and washed by the standard procedure. The purified preinitiation complexes were incubated with selected nucleotides, washed for a second time, then incubated with additional nucleotides. Finally, complexes were challenged to synthesize runoff RNA in the presence of 0.4% Sarkosyl and the remaining nucleoside triphosphates.

When dATP, AMP-PNP, and CTP were present simultaneously in either the first or the second incubation, the complexes became Sarkosyl-resistant, indicating that initiation of RNA synthesis had occurred (Fig. 8, lanes 4 and 7). In contrast, when complexes were incubated with dATP, washed, then incubated with AMP-PNP and CTP, no initiation occurred (lane 9). The presence of dATP in the first incubation did not disrupt the complexes or irreversibly inhibit transcriptional activity, as shown in a control reaction where complexes were incubated with dATP, washed, then incubated with all three substrates (lane 10). Rather, it appears that separate incubation simply failed to satisfy the substrate requirements for the initiation reaction.

Reactions were also tested where complexes were incubated with AMP-PNP and CTP, washed, then incubated with dATP. Again, no initiation was observed (Fig. 8, lane 11). These data indicate that a β,γ-hydrolyzable adenosine nucleotide must be present simultaneously for initiation to occur; the two reactions cannot be uncoupled. This experiment also confirms previous observations that β,γ-hydrolyzable adenosine nucleotides are not required for RNA chain elongation (Fig. 8, lane 7).

**DISCUSSION**

We have characterized the RNA polymerase II-dependent, promoter-specific RNA synthesis initiation reaction using immobilized enzyme complexes. Complex formation requires a warm (30°C) temperature, but was not dependent on the presence of ATP or dATP. Preinitiation complexes were remarkably stable and survived multiple rounds of washing sufficient to remove the great bulk of the protein present in the nuclear extract. These complexes contained all of the transcriptional factors that are required to catalyze the initiation reaction. We cannot rule out the possibility, however, that they lack factors that might be transiently required for complex assembly. The preinitiation complexes were sensitive to 0.25% Sarkosyl, but converted to Sarkosyl-resistant initiated form when incubated with selected nucleotide combinations.

Purification of the transcriptional activity associated with the preinitiation complexes was approximately 300-fold, relative to unfractionated nuclear extracts. It is not expected that a 300-fold purification will be enough to remove all contaminating proteins, and, consistent with this, multiple bands were visible on silver-stained polyacrylamide gels (data not shown). With transcription factors such as Sp1, a purification factor of about 6000-fold, relative to the nuclear extract, was necessary to achieve homogeneity (Briggs et al., 1986). However, the one-step purification reported here represents considerable progress toward purification of the system as a whole, and, even at the present level of purification, it might be possible to visualize individual polypeptides of the transcriptional apparatus, even in the presence of contaminants, by using two-dimensional gel electrophoretic analysis (Franza et al., 1987).

Washing of the preinitiation complexes effectively removed contaminating endogenous nucleotides. This, together with the likelihood that most contaminating nucleotide-metabolizing enzymes have also been removed, should facilitate kinetic analysis by allowing the reaction to be run at defined and constant concentrations of nucleotide substrates.

Previous studies have shown that promoter-specific initiation of transcription by mammalian RNA polymerase II requires a β,γ-hydrolyzable adenosine nucleotide (Bunick et al., 1982; Sawadogo and Roeder, 1984). This requirement is independent of the nucleotides that are needed for incorporation into the first two positions of the RNA transcript and can be satisfied by dATP, which is not ordinarily incorporated into RNA. It has often been supposed that the β,γ-hydrolyzable adenosine nucleotide required for initiation might be the substrate for an ATP phosphohydrolase, that is, it fulfills an "energy requirement" for the initiation reaction.

Because our experiments used template attached to a solid support, we were able to divide the transcription reaction into multiple steps in order to analyze the requirement for β,γ-hydrolyzable adenosine nucleotide. Our results effectively rule out the possibility that the energy of ATP (or dATP) hydrolysis is coupled to a transcription complex assembly step, since transcriptionally competent enzyme complexes can be physically isolated in the absence of endogenous or exogenous ATP (Figs. 6 and 7). Our results also rule out the possibility that the energy of ATP hydrolysis is used to effect a conformational change to a stable, activated form of preinitiation complex. Otherwise, one would have expected that the ATP requirement could have been fulfilled prior to addition of the initiating ribonucleoside triphosphates. In our experiments, the requirement for β,γ-hydrolyzable adenosine nucleotide could not be temporally uncoupled from the requirement for the other substrates (Fig. 8). It remains possible that ATP binds transiently to the preinitiation complex, but dissociates during the washes. This would be consistent with a recent study showing that incubation of preinitiation complexes with ATP provides transient protection against inhibition of transcription by adenosine 5'-O-(3-thiotriphosphate) (Conaway and Conaway, 1988).

There are prior studies suggesting that ATP, in the absence of the other nucleoside triphosphates, is able to bring about a physical change in the preinitiation complex. In some cases, ATP stabilized the complex (Tolunay et al., 1984; Safer et al., 1986), and, in other cases, ATP caused a slow loss of transcriptional activity and a dissociation of protein from the...
template (Cai and Luse, 1987, a and b). The present work does not conflict directly with these observations. We note, however, that ATP may have many effects when incubated with relatively crude fractions. Changes in physical properties induced by incubation with ATP alone may not be directly related to the requirement for ATP for initiation.

The β,γ-hydrolyzable adenosine nucleotide may be a substrate for one or more protein kinases involved in transcription. Sequence analysis of the large subunit of RNA polymerase II shows that there are many potential phosphorylation sites in the COOH-terminal domain (Corden et al., 1985), and the most transcriptionally active form of RNA polymerase II is highly phosphorylated (Dahmus and Kedinger, 1983; Cadena and Dahmus, 1987). Also, in vitro transcription is inhibited by the ATP analogue 5,6-dichloro-1-β-D-ribofuranosyl-
benzimidazole, which also has been shown to inhibit a well-characterized cyclic AMP-independent protein kinase, casein kinase II (Zandomeni et al., 1986). If, in fact, the β,γ-hydrolyzable adenosine nucleotide is the substrate for a protein kinase, both the kinase and its acceptor must be tightly bound to the preinitiation complex. This should allow for ready characterization of both molecules using the DNA-bead approach.

Although the experiments reported here have focused on the process of initiation using well-characterized viral promoters, the DNA-bead system may also have somewhat broader applicability. There are many other promoters that are actively transcribed in vitro by unfractored nuclear extracts, but for which no purified transcription system is available. In some cases, these promoters lack TATA boxes or incorporate novel structural features, suggesting that it may be difficult to adapt existing fractionation schemes. The DNA-bead approach may provide a facile method for studying transcription of these promoters in a purified system. In addition, this system may be useful for obtaining transcriptional activity with extracts from cells or tissues that contain inhibitors of this reaction. Contaminants such as ATPases or ribonucleases should not affect the nucleotide-independent assembly of preinitiation complexes, and, as shown in Fig. 7, such contaminants can be washed away prior to initiation of RNA synthesis.

In vitro transcription generally requires very concentrated reactions. It is thus hard to supplement reactions with saturating amounts of transcription factors that are recovered in dilute form or in high salt buffers following column chromatography. With the immobilized template system, individual factors can be added to the beads and allowed to bind under one set of conditions, after which the buffer can be rapidly exchanged and further assembly allowed to proceed in a small volume with concentrated nuclear extract. This may facilitate experiments that have been difficult in the past.

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Note Added in Proof—Biotin 11-dUTP has been discontinued but may be replaced by biotin-7-dATP (BRL 9590 SA).

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**DNA-Bead Transcription System**

**MATERIALS AND METHODS**

**Buffers**
- Buffer H: 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM dithiothreitol. Buffer D: 50 mM Tris-HCl, pH 7.5, 2% glyc, 10% sucrose, 0.4 M KCl, 5 mM MgCl₂, 0.1 mM EDTA and 2 mM dithiothreitol. TM 0.1 M buffer: 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20% glyc, 12.5 mM MgCl₂, and 0.1 M KCl. TE buffer: 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. TB: 10 mM Tris base, 50 mM NaCl, and 2 mM EDTA. Buffers H and D contained 1 ng/ml of phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, aprotinin, leupeptin, and pepstatin A (Sigma), and TM 0.1 buffer had 0.1 ng/ml of these protease inhibitors.

**Preparation of Cells and Nuclei Extracts**
- HEK cells were grown at 37°C to a density of 2.5 × 10⁶ cells/ml in spinner culture using Joklik minimum essential medium supplemented with 5% calf serum, non-essential amino acids and penicillin. To prepare nuclei extracts, cells were harvested by centrifugation and washed with phosphate-buffered saline containing 1 g/l MgCl₂. The pellets were resuspended in 4 × packed cell volumes (PCV) of buffer H and incubated on ice for 10 minutes. Cells were lysed by sonication in a Dounce B homogenizer. All subsequent manipulations were performed at 4°C except where indicated. The lysed cells were centrifuged at 3,000 rpm for 10 minutes and the pelleted material was resuspended in 4 PCV of buffer H, incubated for 30 minutes on ice, and centrifuged in a Beckman SW 27 rotor at 21,000 g for 1.5 hours. The supernatant (0.5 ml) gtt was added to the supernatant, which was then dialyzed on ice for 1 hour and centrifuged at 15,000 rpm for 1 hour. The supernatant was resuspended in TM 0.1 buffer (0.5 ml in 10 ml of 1% Triton-X 100). Nuclei were isolated by centrifugation at 10,000 rpm in a SW 27 rotor for 1 hour. The supernatant was aliquoted, frozen in liquid nitrogen, and stored at -70°C.

**DNA templates**
- The Adenovirus 2 major late (AdMLP) and SV40 early (SV40EP) promoters were cloned into pUC vectors and designated pAdMLP and pSV40EP, respectively. pAdMLP, viral DNA sequences extend from nucleotide 5700 to 6099 (1751 to 320, relative to the RNA start). In pSV40EP, viral DNA sequences extend from nucleotides 103 to 571 (approximately 1751 to 320, relative to the RNA start). Promoter-containing DNA fragments were biotinylated and isolated as follows: plasmid DNA were linearized with Hind III (pAdMLP) or Eco RI (pSV40EP), which cut at unique sites in the vector DNA shortly upstream of the respective promoter regions.

**AD Major Late Promoter (pAdMLP 439 bp fragment)**

**SV40 Early Promoter (pSV40EP 373 bp fragment)**

**Telomerase activity assays**
- The 3′-end overhangs were filled in using the Klenow fragment of DNA polymerase in the presence of 10 μM thymine-11-deoxy (a 5′-deoxy analog with the thymine at the 5′-position of the pyrimidine base by an 11-atom linker, BRL, 90509), and 100 μM each of dATP, dGTP and dCTP (Pharmacia) as described (Neton et al., 1995). These reactions were evacuated with phenol and chloroform (Maniatis et al., 1982, and the DNA was recovered by ethanol precipitation, dried in vacuo, and digested with Pvu II, which cuts at a unique site in the vector DNA downstream of each promoter region. The biotinylated promoter fragments of 439 bp (pAdMLP) or 373 bp (pSV40EP) were separated from other fragments by polyacrylamide gel electrophoresis, identified by ultraviolet shadowing, and electroeluted from gel slices (Maniatis et al., 1982). Following electroelution, the samples were filtered, concentrated by repeated extraction with n-butanol, and extracted sequentially with water-saturated ether, phenol, and chloroform. The DNA was precipitated and washed with ether, dried in vacuo, resuspended in TE buffer, and quantitated by spectrophotometry at 260 nm.

The incorporation of biotin-11-deoxy into DNA fragments was essentially quantitative, as determined by an electrophoretic mobility shift assay. DNA was incubated in the presence or absence of streptavidin, a bacterial protein with an affinity for biotin. Biotinylated DNA fragments were retained in their mobility when incubated with streptavidin, while non-biotinylated DNA had the same mobility whether or not streptavidin was present (Fig. 2). Internal cleavage of biotinylated AdMLP DNA revealed that only the DNA terminus upstream from the transcriptional cap site was biotinylated, as expected [data not shown].

**DNA-Bead Matrix**
- Purified, biotinylated DNA fragments (10-100 μg) in 5 ml TE buffer were added to 1 ml (packed volume) of thymine-11-deoxy agarose beads with 1.2 mg streptavidin covalently linked via an 11-atom phosphoramidite spacer (SA) (THK) and incubated at 4°C for 4 hr at room temperature. The beads were washed sequentially with 1 ml KO and TE buffer. Supernatants from these washes were analyzed by ultraviolet spectrophotometry at 260 nm for the presence of unbound DNA. In general, greater than 90% of the DNA was associated with the bead matrix. After washing, the DNA beads were resuspended in 1 ml KO buffer and stored at 4°C. Immediately before use, DNA beads were washed 3 times with 1 ml TE buffer.

**In vitro transcription reactions**
- Standard in vitro transcription reactions contained 200,000 ng of DNA template coupled to beads, 15-20 μl of heparin extract, and 5.5 X (final) TM 0.1 buffer in a final volume of 50 μl. The reactions were assembled on ice and incubated at 37°C for 30-60 minutes to allow preparation of complex formation. This was followed by repeated washing at 4°C with 0.5 ml volumes of 0.5 X TM 0.1 buffer. Washed DNA beads were resuspended in 0.5 X TM 0.1 buffer to a final volume of 45-48 μl to initiate RNA synthesis. The following were added: ATP and GTP to a final concentration of 0.5 μM each, 10 μCi per reaction of [3H]UTP, and unlabeled GTP to a final concentration of 20-50 μM (unlabeled nucleoside 5′-triphosphates, Pharmacia, labelled-nucleoside 5′-triphosphates, New England Nuclear). In some experiments AMP-PNP (adenosine monophosphate) and ITP (inosine triphosphate) were substituted for ATP.

Transcription reactions were incubated at 37°C for 2 hr in 5 ml to initiate RNA synthesis. UTP and Inositol (N-tosylcarboxamidinophosphoric acid, Sigma) were added to final concentrations of 400-800 μM and 0.1%, respectively, and the incubation was continued for 30 min, to allow a single round of RNA chain elongation. The reaction was terminated by the addition of 90 μl of 10% SDS, 0.2 M EDTA, 0.2 M NaCl and 240 μg/ml yeast RNA. After extraction with phenol and chloroform the nucleic acids were precipitated by addition of 5 M ammonium acetate in ethanol, mixed in a dry ice-ethanol bath for 5 min, collected by microcentrifugation, washed with 20% ethanol, and dried in vacuo. Pellets were suspended in 15 μl of gel loading dye (7.5 M urea, 10 mM Tris- HCl, pH 7.5, 1 mM EDTA, 10% sucrose with 0.02% of bromophenol blue and xylene cyanol) heated at 70°C for 10 minutes and fractionated on 43% polyacrylamide gels containing 7.5 M urea and 0.1% SDS in 1 x TBE buffer. After electrophoresis the gels were dried in vacuo and exposed to X-ray film.

**Protein Analysis**
- Samples were analyzed by SDS-polyacrylamide gel electrophoresis using a 3% polyacrylamide stacking gel and a 5-15% linear gradient running gel (Laemmli, 1970). Following electrophoresis, the gels were stained with Coomassie Blue (Bio-Rad), destained, and dried in vacuo. The amount of thymine-11-deoxy was determined by densitometry at 683 nm using an LKB Ultrascan XL densitometer. Densitometry scans were excluded, weighed, and compared to determine the relative amounts of protein in different samples.