Factors Involved in Specific Transcription by Mammalian RNA Polymerase II

ROLE OF FACTORS IID AND MLTF IN TRANSCRIPTION FROM THE ADENOVIRUS MAJOR LATE AND IVa2 PROMOTERS*

Juan Carcamo, Sergio Lobos, Alejandro Merino, Leonard Buckbinder‡, Roberto Weinmann, Venkatachala Natarajan, and Danny Reinberg§

From the Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

The role of the adenovirus major late upstream transcription factor (MLTF) in transcription from the adenovirus major late and the IVa2 promoters was studied. The transcription initiation site of the IVa2 promoter is located 210 nucleotides upstream from the CAP site of the major late promoter. Transcription from these two promoters occurs on different DNA strands. Thus, this divergent transcription suggests that the same factor could simultaneously regulate the expression of two different genes. This was investigated utilizing a reconstituted transcription system *in vitro*. The addition of MLTF to reaction mixtures containing the purified general transcription factors and the major late promoter resulted in a 10–12-fold stimulation of transcription. This stimulation was because of an increase of the stability of the preinitiation complex. MLTF allowed DNA template molecules to undergo multiple rounds of transcription. MLTF also stimulated transcription from the adenovirus-encoded IVa2 promoter. Surprisingly, reconstitution experiments indicated that transcription from the IVa2 promoter which does not have a TATA sequence required all the previously described general transcription factors, including TFIID, the TATA binding protein. The requirement for TFIID was demonstrated by reconstitution experiments as well as by oligonucleotide competition experiments. The implications of this observation are discussed.

The control region for genes transcribed by the RNA polymerase II machinery appears to consist of a complex variety of functional domains of DNA sequences (Breathnach and Chambon, 1981; Dynan and Tjian, 1985). One of these, the TATA box, is usually located 20 to 30 nucleotides upstream from the transcription initiation site (CAP site) and appears to position and the start site of transcription (Breathnach and Chambon, 1981). Other DNA sequence elements important for transcription occur upstream of the TATA box. These upstream elements serve as recognition sites for specific DNA binding proteins whose roles remain to be elucidated (Dynan and Tjian, 1985). It is clear that there are many different upstream promoter elements. Some promoters may contain only one of these, whereas other promoters have been found to contain many of these elements arranged in a mosaic (Dynan and Tjian, 1985). The observation that one particular type of upstream element is present in a number of different promoters indicates either that the same factor may be utilized for the control of expression of different genes (Dynan and Tjian, 1985; McKnight and Tjian, 1987; Lin and Green, 1988; Cortes *et al.*, 1988), or that different factors may be able to recognize the same sequence but induce activation from different promoters (Dorn *et al.*, 1987; Cortes *et al.*, 1988). A third type of control element is the enhancer. This sequence is capable of functioning in an orientation-independent manner and at a great distance from the promoter (Khoury and Gruss, 1983).

It thus appears that differential gene expression can be mediated by the interaction of one or many specific transcription factors with critical DNA element(s) present in the promoter and/or the enhancer (Dynan and Tjian, 1983a; Dynan and Tjian, 1985; McKnight and Tjian, 1987). This interaction could ultimately result in the activation (Dynan and Tjian, 1983b; Lee *et al.*, 1987; Cortes *et al.*, 1988) or repression (Nasmyth *et al.*, 1987) of the expression of the gene. The mosaic arrangement of upstream promoter elements and enhancers suggests that the different specific transcription factors must communicate either directly (by protein-protein interactions) or indirectly (via DNA-mediated interactions) with each other and with those factors that operate by binding to the TATA sequence.

Previous studies have indicated that at least five general transcription factors were required for transcription from constructs derived from the adenovirus major late promoter containing the minimum DNA sequence elements (the TATA box and CAP site) (Reinberg and Roeder, 1987; Reinberg *et al.*, 1987b). It has also been demonstrated that optimal transcription from the major late promoter required, in addition to the general transcription factors, another factor, MLTF[1] (Carthew *et al.*, 1985) (also known as USF (Sawadogo and Roeder, 1985a) or UEF (Miyamoto *et al.*, 1984)) that recognized a specific sequence located upstream of the TATA box. The addition of a partially purified protein fraction containing USF to reaction mixtures reconstituted with partially purified

* This research was supported in part by Grant GM 37120 from the National Institutes of Health and Grants 687-035 and 688-026 from the New Jersey Commission on Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from Kirin Beer Company.

§ Recipient of American Cancer Society Junior Faculty Research Award JFRA-205.

[1] The abbreviations used are: MLTF, major late transcription factor; MLP, major late promoter; TF, transcription factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ad-MLP, adenovirus major late promoter.

general transcription factors and the major late promoter resulted in stimulation of transcription (Sawadogo and Roeder, 1985a). The adenovirus major late transcription factor is present in uninfected HeLa cells, and recognition sites for this factor have been observed in cellular promoters (Chodosh *et al.*, 1987; Carthew *et al.*, 1987; Morgan *et al.*, 1988). Thus, an understanding of the mechanism by which MLTF stimulates transcription from the adenovirus major late promoter may have implications for the regulation of transcription of cellular genes.

The adenovirus major late promoter appears to be one of the simplest promoters. Only two DNA elements located upstream of the initiation site are required for transcription (Hen *et al.*, 1982; Miyamoto *et al.*, 1984; Yu and Manley, 1984). However, it also appears that sequences located at and downstream of the CAP site can participate in regulating transcription (Concino *et al.*, 1984; Mansour *et al.*, 1986; Reinberg *et al.*, 1987a; Jansen-Durr *et al.*, 1988; Cohen *et al.*, 1988). In addition, the MLP-CAP site is located 210 nucleotides upstream from the initiation site of the IVa2 promoter, a non-TATA sequence containing promoter. Transcription from these two promoters utilizes different DNA strands (Baker *et al.*, 1979; Baker and Ziff, 1981). Thus it seemed possible that the divergent transcription from these two promoters could contain common elements that regulate their expression.

We have studied the mechanism by which MLTF stimulates transcription from the adenovirus major late promoter and report here that MLTF also participates in regulating transcription from the IVa2 promoter. Analysis of the factors required for transcription from the IVa2 promoter revealed the surprising result that TFIID, the TATA binding protein, participated in transcription from this TATA-less promoter.

MATERIALS AND METHODS

Plasmid DNA—The construction of the recombinant DNA molecules containing the adenovirus major late promoter, pML(C2AT) and pSmaF, were previously described by Sawadogo and Roeder (1985b) and Weil *et al.* (1979), respectively. The recombinant DNA molecule containing the adenovirus IVa2 promoter (pAd-204) was described by Natarajan *et al.* (1984). This DNA molecule contains a deletion of sequences upstream of −204, relative to the IVa2 CAP site, and resulted in the removal of the MLP-CAP site.

Specific Transcription Reactions—Reactions that measured transcription from the adenovirus major late promoter (40 μl) were as previously described (Flores *et al.*, 1988) and contained 20 mM Hepes buffer, pH 7.9, 8 mM $MgCl_2$, 60 mM KCl, 10 mM ammonium sulfate, 12% (v/v) glycerol, 4 mM dithiothreitol, 0.6 mM ribonucleoside triphosphates (ATP and CTP), 12.5 μM [α-^{32}P]UTP (10,000 cpm/pmol), 2% (w/v) polyethylene glycol 8000, and DNA pML(C2AT) (Sawadogo and Roeder, 1985b) or pSmaF (Weil *et al.*, 1979) which was linearized with the restriction endonuclease *Sma*I, as indicated in the figure legends. Mixtures that received pML(C2AT) DNA were also supplemented with ribonuclease T1 (Sawadogo and Roeder, 1985b). Reactions were supplemented with purified transcription factors: TFIIA (single-stranded DNA agarose fraction, 0.3 μg), TFIIB (single-stranded DNA agarose fraction, 0.2 μg), TFIID (single-stranded DNA agarose fraction, 0.2 μg), TFIIE/TFIIF (Affi-Gel Blue fraction, 0.35 μg), RNA polymerase II (2.8 units), and MLTF (DNA affinity step, 4 μl, or as indicated in the figure legends). Reactions that measured transcription from the adenovirus IVa2 promoter (40 μl) were as described for the major late promoter; however, the KCl concentration was reduced to 45 mM and the polyethylene glycol was omitted. The DNA template (plasmid DNA pAd-204, 12.5 μg/ml (Natarajan *et al.*, 1984)) was linearized with the restriction endonuclease *Hinc*II. Transcription from the IVa2 major initiation site to the *Hinc*II site of plasmid pAd-204 produced an RNA molecule of 654 nucleotides. The factors used in the IVa2 transcription reactions were derived as follows (for detail see Reinberg *et al.*, 1987b). TFIIA (single-stranded DNA agarose fraction, 0.8 μg), TFIIB (single-stranded DNA agarose fraction, 0.12 μg), TFIIE/TFIIF (Sephacryl S-200 fraction, 1.4 μg), TFIID (carboxymethyl cellulose fraction, 0.15 μg), RNA polymerase II (2.8 units), MLTF (DNA affinity step, 4 μl). Transcription reactions were incubated at 30 °C for 75 min, unless otherwise indicated in the figure legends.

The products of the reactions were separated by electrophoresis on 4% polyacrylamide, 7 M urea gels. The amount of radiolabeled nucleotides incorporated into the specific transcripts was determined by excising the labeled band from the dry gel and counting in a scintillation counter.

Purification of MLTF—Nuclear extracts were prepared from 30 liters of HeLa cells grown to a density of 0.8–1.0 × 10^6 cells/ml as previously described (Dignam *et al.*, 1983). Six different preparations of nuclear extracts (total 4590 mg, 540 ml) were pooled and chromatographed on a phosphocellulose column (10 mg of protein/ml of packed resin) equilibrated with buffer C (20 mM Tris-HCl, pH 7.9 (at 4 °C), 20% (v/v) glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) as previously described (Reinberg and Roeder, 1987). The MLTF transcriptional and specific DNA binding activities were present in the phosphocellulose 0.1 M (3.9 mg/ml, 700 ml) and 0.3 M (2.1 mg/ml, 400 ml) KCl washes. This is in agreement with previous studies that detected MLTF activity in the phosphocellulose 0.1 M KCl fractions (Carthew *et al.*, 1985a) as well as in the phosphocellulose 0.3 M KCl wash (Sawadogo and Roeder, 1985a). The further fractionation of either fraction, by chromatography on a DEAE-cellulose column (10 mg of protein/ml resin), resulted in the separation of two forms of MLTF. One form contained only DNA binding activity which was specific for the MLTF recognition site; the other form contained both transcription and DNA binding activities. The transcription activity present in the fractions derived from the phosphocellulose 0.3 M KCl wash was 2–3-fold higher than the one derived from the phosphocellulose 0.1 M KCl wash (data not shown). Further fractionation of the DEAE-cellulose transcriptionally active pool derived from the phosphocellulose 0.3 M KCl wash was performed by DNA affinity chromatography on a column containing multiple copies of the MLTF recognition site (Ad-MLP sequences extending from −73 to −48, relative to the CAP site). The column was prepared as described by Kadonaga and Tjian (1986). A fraction of the DEAE-cellulose pool (6 mg) which was in buffer C containing 0.1 M KCl and 8 mM MgCl was mixed with nonspecific competitor DNAs (poly[d(I-C)], 100 μg/ml, poly[(dA)·(dT)], 100 μg/ml) and polyethylene glycol 8000 to 2% (w/v) final concentration. The mixture was loaded onto a 3-ml column equilibrated with buffer C containing 0.1 M KCl and 8 mM MgCl as previously described by Cortes *et al.* (1988). The column was washed with the above buffer (9 ml/h), and the proteins that bound to the column eluted with successive washes (9 ml) with buffer C containing 8 mM MgCl, 100 μg/ml bovine serum albumin, and 0.2 and 0.5 M KCl. The transcriptionally active fractions (present in the 0.5 M KCl wash) were pooled and further purified by two additional cycles of DNA affinity column chromatography. The conditions for chromatography were as described above; however, the concentration of competitor DNA was reduced to one-half of the original concentration and none for the second and third cycle, respectively.

DNA Binding Assays—DNA binding reactions (30 μl) were carried out under conditions of transcription from the MLP. However, the ribonucleoside triphosphates and the ammonium sulfate were omitted. In addition, reaction mixtures were supplemented with nonspecific competitor DNA (poly[d(I-C)], 15 μg/ml) and a 455-base pair 3′-end-labeled DNA fragment (2–9 ng) originated by digesting pSmaF DNA (Weil *et al.*, 1979) with the restriction endonucleases *Hind*III and *Xho*I. The label was at the *Hind*III site. The products of the reactions were separated by electrophoresis on a 4% polyacrylamide gel which was developed using a low ionic strength buffer (40 mM Tris, 40 mM boric acid, 1 mM EDTA).

DNase I Protection Experiments—The binding of MLTF to the DNA was performed as described in the previous section. After incubation at 30 °C for 60 min, the mixtures received 3 μl of a solution containing 5 mM CaCl and 1 μl of DNase I (Boehringer Mannheim) that was freshly diluted to 10 μg/ml. The mixtures were incubated for an additional 2 min at 25 °C. Maxam and Gilbert G and G + A sequence ladders of the DNA probe were prepared as described and subjected to electrophoresis on urea sequencing gels adjacent to the footprint reactions.

RESULTS

MLTF Stimulates Transcription from the MLP—Optimal transcription from the major late promoter requires, in addition to the TATA box, an upstream element located at about

position −60 (relative to the CAP site). It was shown that this element was recognized by a 46,000-dalton protein, MLTF (Chodosh *et al.*, 1986).

Transcription from a promoter containing a deletion of the MLTF recognition site, as well as that from a wild type promoter in the absence of MLTF, required five general transcription factors (IIA, IIB, IID, IIE, and IIF) in addition to RNA polymerase II (Reinberg and Roeder, 1987; Reinberg *et al.*, 1987b; Flores *et al.*, 1988). These factors operated via the TATA sequence. The addition of HeLa cell nuclear extracts to reaction mixtures reconstituted with saturating amounts of the general transcription factors resulted in stimulation of transcription from the MLP. This functional transcription assay, in combination with a specific DNA binding assay was used to purify MLTF. The purification is described under "Materials and Methods." The last step in the fractionation included chromatography on a DNA affinity column. This step resulted in the isolation of two major polypeptides, one of 46,000 daltons and another of 50,000 daltons (Fig. 1*A*). Both the 46,000- and 50,000-dalton protein appears to coelute with the DNA binding activity (Fig. 1*B*). This was further demonstrated as both polypeptides were capable of binding to the MLTF recognition site after elution and renaturation of the proteins from a polyacrylamide sodium dodecyl sulfate gel (data not shown). This is in agreement with the studies of Chodosh *et al.* (1986), which demonstrated that the DNA binding activity associated with MLTF was contained in a polypeptide of 46,000 daltons. The nature of the 50,000-dalton polypeptide present in our preparations is unknown. It is possible that it is a modified form of the 46,000-dalton protein, but this remains to be further investigated. Interestingly, when cruder fractions of earlier steps of purification were analyzed by DNase I footprinting experiments, factors that specifically bound to DNA sequences present downstream of the MLP initiation site were detected (Fig. 1*B*, *lane 2*). The effect of these factors (DF-1, DF-2, and DF-3) in transcription from the MLP have been independently analyzed and will not be discussed further here.[2]

The addition of affinity purified MLTF to reactions reconstituted with the general factors resulted in stimulation of transcription. The rate of the reaction was affected: reactions carried out in the presence of MLTF showed a 5-min lag, whereas reactions carried out in the absence of MLTF showed a 10-min lag (Fig. 2). Also, after a 5-min lag period, reactions that received MLTF proceeded at an increased rate (Fig. 2). The observed effect was specific to MLTF, since reactions carried out with DNA templates in which the MLTF recognition site was deleted followed kinetics similar to reactions in which MLTF protein was omitted (data not shown, see below). Because MLTF is a specific DNA binding protein and

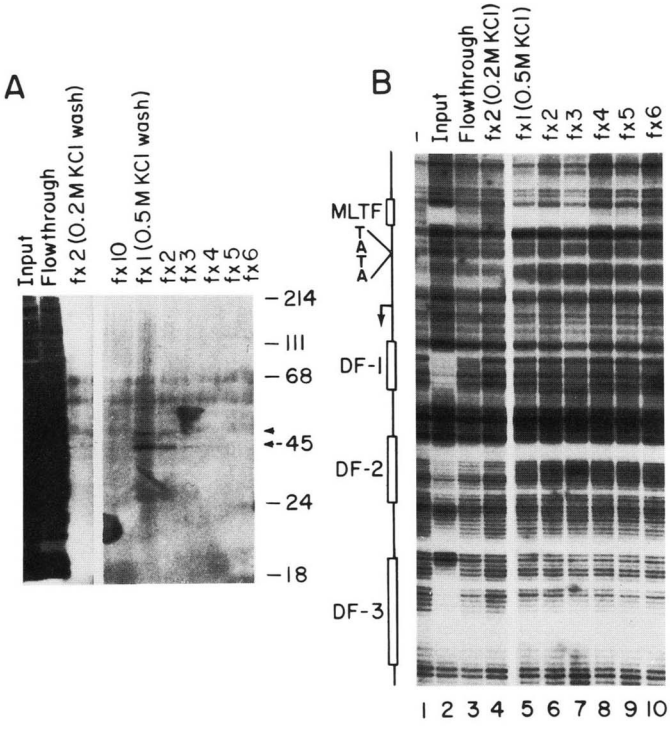

FIG. 1. **Analysis of MLTF by electrophoresis on a polyacrylamide-sodium dodecyl sulfate gel.** Aliquots (50 μl) of the different fractions of a representative DNA affinity chromatographic step were separated by electrophoresis on a 10% polyacrylamide-sodium dodecyl sulfate gel (*panel A*) and visualized by silver staining. The lane labeled *Input* represents the polypeptides present in the DEAE-cellulose fraction (0.5 μg of protein) that was applied to the affinity column. *Flowthrough* represents the unbound polypeptides present in 0.5 μg of protein from the DNA affinity column. In *panel B* the DNA binding activity present in the different fractions (in 10 μl) was analyzed by a DNase I footprinting experiment. The conditions of the assay and analysis of the products were as indicated under "Materials and Methods." The nucleotides protected from DNase I digestion were as follow: MLTF footprint extended from −49 to −67, DF-1 footprint extended from nucleotide +24 to +50, DF-2 footprint extended from +64 to +84, DF-3 footprint extended from +91 to +107. The fractions of this representative step of purification were devoid of transcription activity; this is probably because the bovine serum albumin protein carrier was omitted from the salt washes (see "Materials and Methods") to allow analysis of the polypeptides.

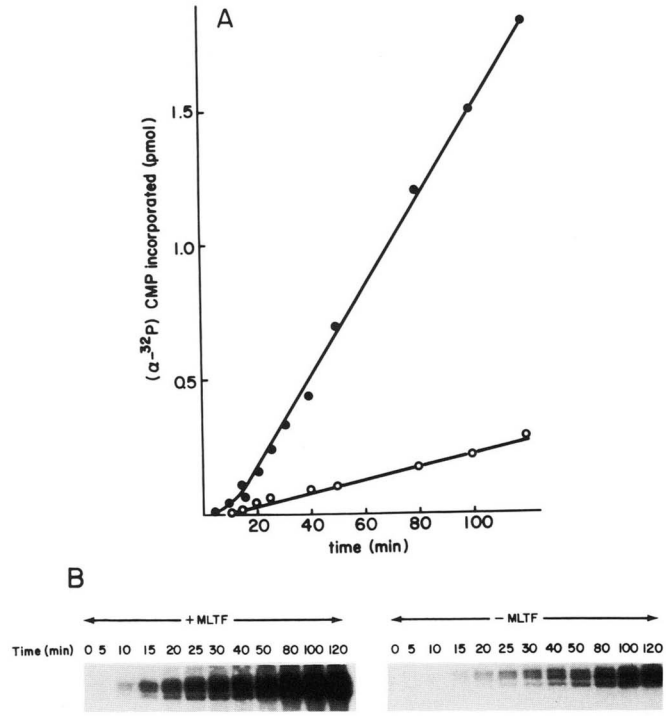

FIG. 2. **Effect of MLTF on the time course of transcription from the MLP.** Reactions containing the general transcription factors, RNA polymerase II and a plasmid DNA (2.5 μg/ml) containing the MLP (pML (C2AT) (Sawadogo and Roeder, 1985b)) were incubated under transcription conditions with and without MLTF for different periods of time, as indicated in the figure. The products of the reactions were separated by electrophoresis on a 4% polyacrylamide-urea gel (*panel B*). The amount of radiolabeled nucleotides incorporated into the specific transcript was determined by excising the bands of the dry gel and counting in a scintillation counter (*panel A*).

[2] J. Carcamo and D. Reinberg, manuscript in preparation.

previous studies have indicated that this factor and the TATA binding protein cooperated to produce a stable DNA-protein complex at the MLP (Sawadogo and Roeder, 1985a), we analyzed whether MLTF affected the formation of a preinitiation complex. The transcription reaction was divided into two steps, by the addition of Sarkosyl (Hawley and Roeder, 1985), as described in the lower part of Fig. 3. Transcription factors and a DNA template containing the MLP (pML(C2AT) (Sawadogo and Roeder, 1985b)) were incubated with and without MLTF for different periods of time. Nucleoside triphosphates were then added. This was followed, 1 min later, by the addition of Sarkosyl. Reaction mixtures were then incubated an additional 30 min. The addition of MLTF affected the rate as well as the amount of complex formed. The overall stimulation was 2-fold under the conditions of this experiment where reinitiation was blocked by the addition of Sarkosyl.

The stimulation observed by MLTF was dependent on the concentration of factor as well as on the concentration of DNA template added to the reaction. A stoichiometric relationship between MLTF and the DNA (containing the MLP) was observed (data not shown). This result is in agreement with the studies of others (Sawadogo and Roeder, 1985a; Lennard and Egly, 1987).

MLTF Affected the Stability of a Preinitiation Complex— The results described in the previous section suggested that MLTF was capable of stimulating transcription by increasing the number of preinitiation complexes formed (Fig. 3). This effect could be explained if MLTF increased the stability of the preinitiation complex at the MLP. This possibility was analyzed. A DNA molecule containing the Ad-MLP directing transcription of a 387-nucleotide-long synthetic polydeoxynucleotide with no cytidilic residues in the transcribed strand (pML(C2AT) (Sawadogo and Roeder, 1985b)) was incubated with the transcription factors and RNA polymerase II, with and without MLTF (Fig. 4). After a 30-min incubation period

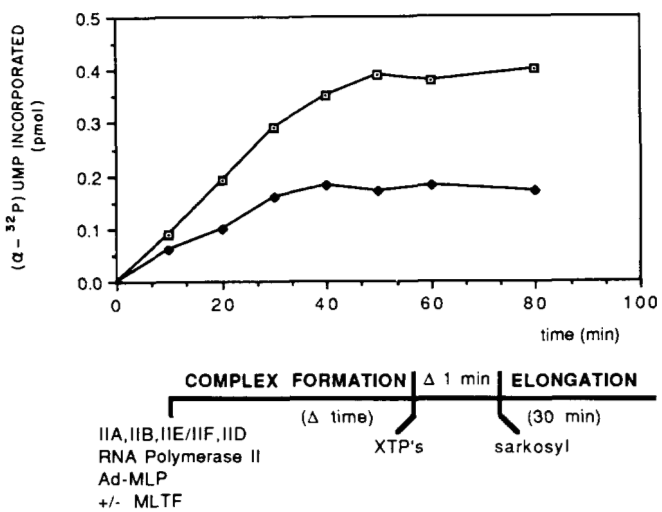

FIG. 3. **The effect of MLTF in the formation of a preinitiation complex at the MLP.** Two 400-μl reaction mixtures that contained the general transcription factors, RNA polymerase II and a plasmid DNA containing the MLP (pML(C2AT) (2.5 μg/ml) were incubated with and without MLTF. After different periods of incubation, as indicated in the lower part of the figure, an aliquot (30 μl) was removed, nucleotides were added, and the reaction was further incubated for 1 min. Sarkosyl was then added (to 0.02%) and the reaction incubated for an additional 30 min. Products of the reaction were separated on a 4% polyacrylamide, 7 M urea gel. The bands corresponding to the specific transcript were excised from a dry gel and counted.

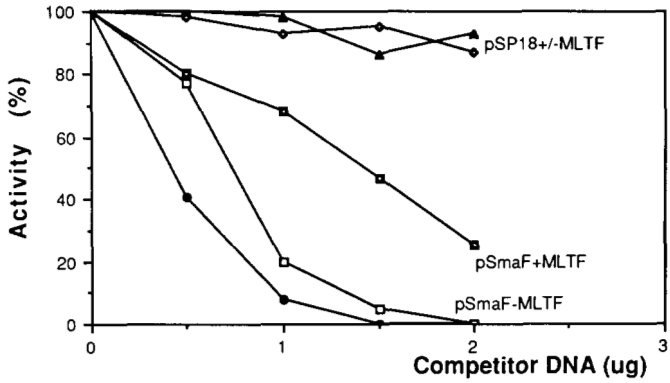

FIG. 4. **MLTF affects the stability of the preinitiation complex.** DNA molecules containing the Ad-MLP (pML(C2AT) (10 μg/ml)) were incubated with the general transcription factors, with (◆——◆, ■——■) and without (▲——▲, □——□) MLTF, to allow the formation of preinitiation complexes. After 30 min of incubation, increasing amounts of nonspecific competitor DNA (pSP18) or a DNA containing the MLP (pSmaF) were added, as indicated in the figure. Another set of tubes contained the specific competitor DNA together with MLTF from the beginning of the first incubation (●——●). The addition of competitor DNA was followed, after 15 min of incubation, by the ribonucleoside triphosphates ATP, CTP, and UTP in addition to ribonuclease T1, and reactions were elongated for 30 min. Products of the reaction were separated on polyacrylamide-urea gels. Bands corresponding to specific transcripts were excised from the dry gel and counted.

where preinitiation complexes were allowed to form, increasing amounts of competitor DNA (nonspecific DNA (pSP-18 (BRL)) or a DNA that contained the MLP (pSmaF (Weil *et al.*, 1979)) were added. This was followed by the addition of the ribonucleoside triphosphates (ATP, CTP, and UTP) and ribonuclease T1, and the reactions were incubated for 30 min. Since reactions were carried out in the absence of GTP, only transcripts from the pML(C2AT) template were observed. The inclusion of ribonuclease T1 in the transcription reaction mixture degrades RNA molecules originating from pSmaF (the second template used to challenge the stability of the complex) that could be produced as a consequence of GTP contamination. The addition of pSmaF DNA (competitor DNA) resulted in inhibition of transcription. However, significant differences were observed when MLTF was present in the reactions. The addition of 1 μg of pSmaF DNA, after 30 min of preincubation, resulted in 80% inhibition, whereas only a 25% inhibition was observed if the reaction mixtures contained MLTF (Fig. 4). The addition of 1.5 μg of pSmaF DNA resulted in 90% inhibition of transcription when MLTF was omitted, whereas only a 50% inhibition was observed when MLTF was present (Fig. 4). The low levels of inhibition by the competitor DNA, in the presence of MLTF, were dependent on preincubation. Inclusion of pSmaF DNA to reaction mixtures which were not preincubated with the general factors and MLTF resulted in a drastic inhibition of transcription from the pML DNA template; 0.5 μg of DNA produced 60% inhibition (Fig. 4). The addition of nonspecific DNA (pSP18), after a 30-min incubation, did not inhibit the reaction; this was independent of the presence of MLTF.

The above results strongly suggest that MLTF stabilizes the preinitiation complex. This observation is in agreement with the studies of Sawadogo and Roeder (1985a), which demonstrated using DNase I footprinting experiments that MLTF and the TATA binding protein cooperated to produce a stable DNA-protein interaction.

MLTF Stimulates the Formation of a Committed Complex— It was previously indicated that the formation of a committed complex at the Ad-MLP (defined by its resistance to low

concentrations of Sarkosyl (Hawley and Roeder, 1985)) required TFIIA and TFIID, the TATA binding protein (Reinberg *et al.*, 1987a). It was also demonstrated that RNA polymerase II increased the amount of complexes resistant to low concentrations of Sarkosyl (Reinberg *et al.*, 1987a). Because in the earlier experiments the TFIIA protein fraction used (Sephacryl S-200) also contained MLTF (see "Materials and Methods"), we restudied the effect of TFIIA and analyzed the role of MLTF in the formation of a committed complex.

The protocol described in the legend to Fig. 5 was followed. In agreement with the early observation, the incubation of the general transcription factors and RNA polymerase II with the Ad-MLP, prior to the addition of Sarkosyl, resulted in the formation of a complex that was capable of undergoing transcription when nucleoside triphosphates were added (Fig. 5, *lanes 1* and *11*). The addition of MLTF to reaction mixtures incubated in this manner resulted in an approximately 2-fold stimulation of transcription (*lanes 2* and *12*). This stimulation was consistent with the results presented in Fig. 3, which indicated that MLTF stimulated single round transcription by a factor of 2. Furthermore, and in agreement with earlier observations (Reinberg *et al.*, 1987a), the omission of TFIIB and TFIIE/TFIIF from the preincubation had no effect on the formation of a committed complex (*lane 3*); however, the omission of RNA polymerase II resulted in a decrease in the amount of Sarkosyl-resistant complex formed (*lane 4*). Contrary to our previous observation indicating that the incubation of only TFIIA and TFIID with the Ad-MLP, prior to Sarkosyl addition, was capable of producing low amounts of Sarkosyl-resistant complex, we were not able to observe such a complex (Fig. 5, *lane 7*). However, the inclusion of RNA polymerase II (*lane 5*) or MLTF (*lane 4*) to a reaction mixture that contained TFIIA, TFIID, and the MLP produced low amounts of Sarkosyl-resistant complexes. These results suggested that RNA polymerase II and MLTF had a similar effect on the production of a committed complex, probably the stabilization of a preformed complex. The addition of MLTF and RNA polymerase II, to a reaction that contained TFIIA, TFIID, and the MLP, resulted in an increase in the amount of Sarkosyl-resistant complex formed (*lane 3*). The omission of TFIIA resulted in the formation of a drastically reduced amount of committed complex (*lane 6*). This result may suggest that MLTF can partially overcome the requirements for TFIIA or that some of the factors were contaminated with trace amounts of TFIIA. The incubation of TFIID with MLTF (*lane 9*) or RNA polymerase II (*lane 10*), in addition to the MLP prior to Sarkosyl addition, failed to produce any detectable levels of committed complex.

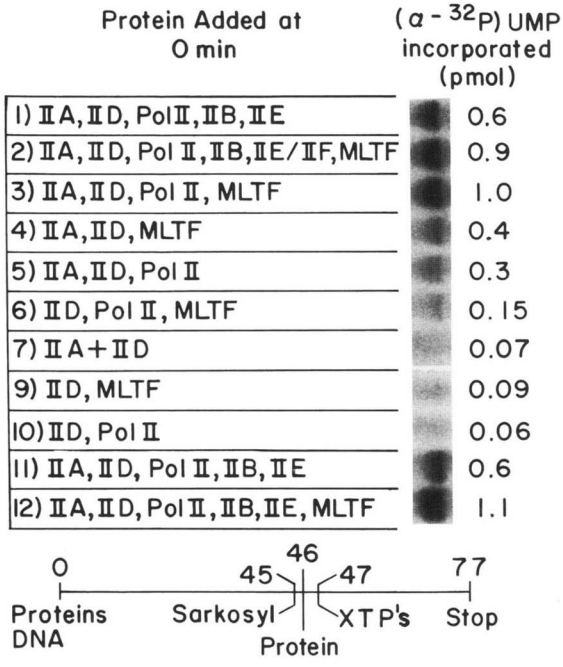

FIG. 5. **MLTF stimulates the formation of a committed complex.** Reaction mixtures (60 μl) were carried out as described in the *diagram* in the *lower part* of the figure. Reactions contained the general transcription factors, RNA polymerase II, MLTF, or a subset of these as indicated, and a DNA containing the major late promoter (pML(C2AT) (83 μg/ml)), were incubated at 30 °C for 45 min before Sarkosyl (to 0.02% w/v) was added. After 1 min, the protein(s) omitted from the first incubation were added; after another minute, the ribonucleoside triphosphates were added and the incubation continued at 30 °C. After 30 min the reactions were processed for product analysis as described. Products of the reaction were separated by electrophoresis on a 4% polyacrylamide, 7 M urea gel. Bands were excised from the dry gel, and the amount of labeled nucleotide incorporated was determined.

These data together with those presented in Fig. 4 indicated that MLTF increased the stability of a committed complex. Since the incubation of TFIID, TFIIA, and RNA polymerase II with the MLP (*lane 5*), resulted in low levels of committed complex, an absolute requirement for MLTF in the formation of such a complex seems unlikely. To rule out the possibility that trace amounts of MLTF were present as contaminants in the TFIIA, TFIID, or RNA polymerase II preparations, we performed DNA binding assays using the gel retardation method (data not shown). The most logical interpretation of these results is that both TFIID and TFIIA are required for the formation of an initial complex at the MLP. This complex is sensitive to Sarkosyl, but can be converted to a partially resistant from by RNA polymerase II or MLTF. A complex fully resistant to Sarkosyl requires TFIIA, TFIID, MLTF, and RNA polymerase II. The requirement of polymerase in such a complex is probably artifactual because of an effect of Sarkosyl on the association of the polymerase with the complex (Reinberg *et al.*, 1987a).

Similar results were obtained by Davidson *et al.* (1983) and Fire *et al.* (1984). Their studies indicated that the formation of an initial complex at the major late promoter required two protein fractions which are equivalent to TFIIA and TFIID; however, their experiments were performed with crude preparations of TFIIA; thus it is possible that the complex also contained MLTF.

MLTF Stimulates Multiple Rounds of Transcription—The above studies indicated that MLTF was capable of stimulating the overall transcription reaction 10-fold (Fig. 2). This stimulation was because of both an increase of the rate of the reaction as well as an increase in the amount of transcript produced. Furthermore, when transcription reactions were studied under conditions in which initiation was limited to one round by Sarkosyl addition, MLTF only stimulated transcription 2-fold. These results suggested that MLTF also affected, directly or indirectly, a step after the formation of a preinitiation complex.

It has previously been demonstrated that the formation of a committed complex at the MLP served as an entry site for RNA polymerase II into the transcription cycle (Reinberg *et al.*, 1987a; also see Fig. 5). Reactions carried out in the absence of MLTF (or with limiting amounts of MLTF (Reinberg *et al.*, 1987a)) formed a stable committed complex only when RNA polymerase II was present (see Fig. 5); thus, it is pertinent to postulate that when the polymerase leaves the complex, to undergo elongation, the committed complex (now only containing TFIIA and TFIID) dissociates. However, if

MLTF stabilizes the formation of a committed complex; when RNA polymerase translocates during elongation, the committed complex should remain at the promoter (containing TFIID, TFIIA, and MLTF) where it would be free to be recognized by a second polymerase II molecule.

The addition of MLTF resulted in a stimulation of the overall transcription reaction (Fig. 2). This stimulation could be correlated with an increase in the amount of full-size transcripts (Fig. 2); however, shorter RNA molecules, which were dependent on the presence of MLTF and separated by a periodicity were also observed as products (Fig. 6, *A* and *B*, *lanes 2–4*, also see Sawadogo and Roeder (1985a)). The shorter RNA molecules, as well as the full size RNA molecules, appeared as a triplet (see Fig. 6*B*). The meaning of these triplets at present is unknown; it is possible that they originated by GTP contamination and incomplete digestion with endonuclease T1, as the end of the synthetic polydeoxynucleotide chain contains 3 cytidilic residues as part of a recognition site for the *Sma*I endonuclease (see Fig. 6*C*).

A possible interpretation of these results, as initially postulated by Sawadogo and Roeder (1985a), is that the DNA templates in reactions carried out in the presence of MLTF could undergo multiple rounds of transcription. Since the MLP directed transcription of a synthetic 387 polydeoxynucleotide lacking cytidilic residues in the transcribed strand (pML(C2AT) (Sawadogo and Roeder, 1985b)), and reactions were carried out using a circular DNA molecule in the absence of GTP, then when the elongating machinery arrives to the end of the synthetic sequences and encounters the first C residue in the plasmid DNA sequences (see Fig. 6*C*), RNA polymerase II stops due to the absence of GTP. The difference in sizes (approximately 40 nucleotides) of each of the small transcripts probably represents the length of the DNA covered by each paused RNA polymerase II molecule.

In order to analyze whether the small RNA molecules produced in the presence of MLTF resulted from reinitiation, the experiment described in the lower part of Fig. 6*D* was performed. Preinitiation complexes at the major late promoter were formed with and without MLTF. After 45 min of incubation Sarkosyl was added to prevent reinitiation (Hawley and Roeder, 1985). This was followed, 1 min later, by the addition of nucleotides. Reactions were elongated for 30 min. The addition of MLTF resulted in a stimulation of transcription (compares *lanes 1* and *3* with *4* and *5*, respectively). The addition of Sarkosyl after preincubation did not affect the MLTF mediated stimulation of transcription (compare *lanes 3* and *4*); however, it did inhibit the production of the small RNA molecules (compare *lanes 2* and *4*). Reactions carried out in the absence of MLTF (*lanes 1* and *3*) yielded only full size transcripts, whereas reactions carried out in the presence of MLTF but in the absence of Sarkosyl produced full size transcripts as well as a set of bands that were shorter than the full size transcript. The shorter bands were sensitive to Sarkosyl.

These results strongly suggest that MLTF permits more than one round of transcription per DNA template. Interestingly, an unequal distribution of labeling was observed between the full size transcript and the shorter transcripts (Fig.

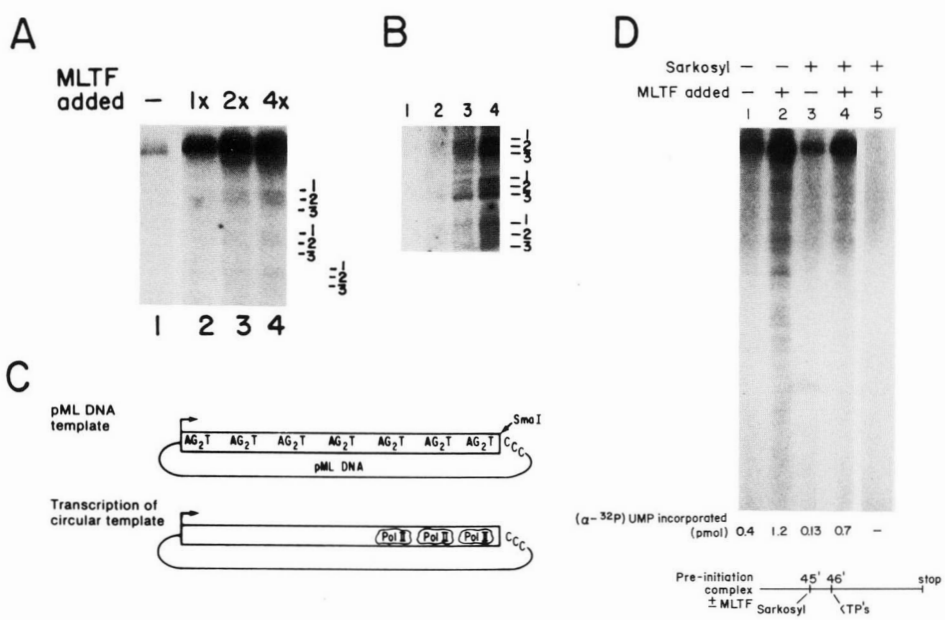

FIG. 6. **The effect of MLTF on transcription from a circular DNA template.** *Panel A,* transcription reactions were as described under "Materials and Methods" and contained the general transcription factors, RNA polymerase II and increasing amounts of affinity purified MLTF, as indicated in the figure. In addition, a circular DNA template (pML(C2AT) (2.5 μg/ml)) in which the major late promoter directed transcription of a 387-base pair synthetic DNA fragment lacking cytidilic residues in the transcribed strand was used (Sawadogo and Roeder, 1985b; also see *panel C*). The products of the reactions were analyzed as described. The amount of [α-^{32}P]UMP incorporated into the specific transcripts was determined by excising each lane from the dry gel. The *X* at the top of the panel indicated different amounts of MLTF added. The exact concentration of MLTF added is unknown because the DNA affinity-purified fraction contained bovine serum albumin (100 μg/ml). This is required to preserve the transcriptional activity of MLTF. *Panel B,* over-exposure of the lower part of the gel shown in *panel A*; *panel C,* schematic representation of the pML(C2AT) DNA template and its transcription. For details see text: *Panel D,* the effect of Sarkosyl on MLTF-mediated stimulation of transcription from the MLP. Reaction mixtures were as described in the legend to Fig. 5 and outlined in the *lower part* of the *figure.* After 45 min of incubation, Sarkosyl and nucleotides were added as indicated in the figure. Reactions were elongated for 30 min, and the products were separated by electrophoresis on a 4% polyacrylamide-urea gel.

6, *A*, *B*, and *D*). This suggests that not every DNA molecule that formed a preinitiation complex and underwent one round of transcription was capable of reinitiation. This could be explained if not every MLTF molecule that bound to its cognate site was transcriptionally active and capable of stabilizing a productive interaction with the other transcription factors. This possibility is in agreement with the observation that at least two populations of MLTF could be observed during purification, one containing transcription and DNA binding activities and another containing only the latter (see "Materials and Methods").

MLTF Stimulates Transcription from the Adenovirus IVa2 Promoter—The initiation site of the IVa2 promoter, a non-TATA sequence containing promoter, is located 210 nucleotides upstream from the MLP-CAP site. The arrangement of the transcription initiation sites and the fact that transcription from these two promoters utilizes different DNA strands, offers the possibility that they may share upstream regulatory elements (see Fig. 11*A* and Natarajan *et al.*, 1987). Furthermore, the studies of Lennard and Egly (1987) demonstrated that, when the orientation of the MLTF recognition site was inverted, it still was functional for transcription from the MLP. This observation further suggested that the MLTF could regulate transcription from both promoters. In order to study the possible role of MLTF in transcription from the IVa2 promoter, we attempted to reconstitute transcription from this promoter.

Previous studies have indicated that sequences around the MLP-CAP site had an inhibitory effect on transcription from the IVa2 promoter (Natarajan *et al.*, 1984, 1985). We have reproduced this observation and interestingly, the inhibitory effect of the MLP-CAP site and surrounding sequences on IVa2 transcription was only observed when the two promoters were linked (in cis) (data not shown). Thus, in our studies on transcription from the IVa2 promoter we utilized a DNA plasmid from which sequences upstream of −204 (relative to the IVa2 CAP site) were deleted (deletion of the MLP-CAP site). Transcription from the IVa2 promoter required all the previously described general factors (Fig. 7). The omission of TFIIA, RNA polymerase II, TFIIE/TFIIF, or TFIIB from the reconstituted system resulted in low or no transcription (*lanes 1*, *5*, *6*, and *10*, respectively). Furthermore, the levels of transcription from the IVa2 promoter were directly proportional to the amount of each of the general factors added to the reaction (Fig. 7). Interestingly and surprisingly, transcription from this TATA-less promoter in the reconstituted system also required TFIID, the TATA binding protein (Fig. 8*A*, *lanes 1–5*, and see below). MLTF was also required for optimal levels of transcription from the IVa2 promoter (Fig. 9). Transcription from the IVa2 promoter in the absence of MLTF was observed (Fig. 9*A*, *lane 1*, and Fig. 9*B*). The addition of MLTF resulted in stimulation of transcription. The stimulation was proportional to the amount of MLTF added (Fig. 9, *A* and *B*) and dependent on TFIID (Fig. 9*B*). The highest concentration of MLTF added produced 8-fold stimulation (Fig. 9*B*).

The requirement of TFIID and MLTF on transcription from the IVa2 promoter was further analyzed by oligonucleotide competition experiments. The addition of different amounts of oligonucleotides containing the MLTF recognition site to a partially reconstituted transcription system inhibited transcription from the IVa2 and ML promoters (Fig. 10, compare *lanes 8* with *12–14* and *lane 1* with *5–7*, respectively). The inhibition was specific for the IVa2 and ML promoters, as inclusion in the same reaction mixture of a second promoter which lacks the MLTF recognition site, the

IIB (μg)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.06	—
IIE/IIF (μg)	1.4	1.4	1.4	1.4	1.4	—	0.7	1.4	1.4	1.4
POl II (ng)	36	36	36	36	—	36	36	36	36	36
IIA (μg)	—	0.2	0.4	0.8	0.8	0.8	0.8	0.8	0.8	0.8
	1	2	3	4	5	6	7	8	9	10

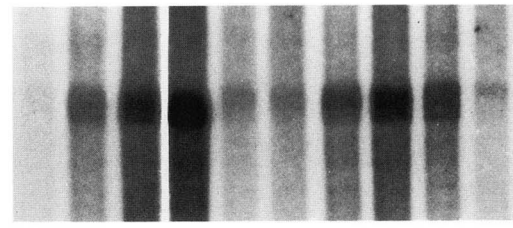

FIG. 7. **Factor requirement for transcription from the IVa2 promoter.** Reaction mixtures were as described under "Materials and Methods" and contained a plasmid DNA containing the IVa2 promoter (pAd −204 (Natarajan *et al.*, 1984) (10 μg/ml)), TFIID (CM-cellulose fraction, 0.15 μg), affinity-purified MLTF (4-μl), and the general transcription factors IIA, IIB, IIE/IIF, and RNA polymerase II as indicated in the figure and purified as described under "Materials and Methods." Reactions were incubated at 30 °C for 90 min. Products were separated by electrophoresis on a 4% polyacrylamide, 7 M urea gel.

Eiv promoter, was not affected. Conversely, the addition of oligonucleotides containing the recognition site for a factor that is specifically required for transcription from the Eiv promoter, EivF (Cortes *et al.*, 1988), had no effect on transcription from the IVa2 promoter, but inhibited transcription from the Eiv promoter (Fig. 8, *lanes 18* and *19*). Consistent with the observation that TFIID was required for transcription from the IVa2 promoter, the addition of oligonucleotides containing the MLP-TATA sequence, extending from −174 to −199 (relative to the IVa2 CAP site) resulted in inhibition of transcription from the IVa2, ML, and Eiv promoters (*lanes 2–4* and *9–11*). This inhibition appears to be specific since the addition of an oligonucleotide containing "nonimportant" sequences from the IVa2 promoter, extending from −100 to −130, had no effect on transcription from the IVa2 or Eiv promoters (*lanes 15–17*). In order to further analyze the specificity of inhibition of the oligonucleotides containing the MLP TATA sequence on transcription from the TATA-less promoter, the experiment described on the lower part of Fig. 8*A* was followed. A plasmid DNA containing the IVa2 promoter together with either a plasmid DNA containing the MLP (with sequences extending from −56 to +33 (pD-139, Reinberg and Roeder, 1987), *lanes 18–23*), or the Eiv promoter (with sequences extending from −46 to +70 (Leza and Hearing, 1988), *lanes 12–17*), or an Eiv promoter without TATA box (with sequences extending from −11 to +70 (Leza and Hearing, 1988), *lanes 6–11*), were mixed with TFIID and all the other factors required for optimal transcription from the IVa2 promoter. After 10-min incubation, mixtures containing the highest concentration of competitor DNA (pD-139 or pE4) were supplemented with increasing amounts of TFIID, as indicated on the figure. After another 10 min of incubation, reactions received ribonucleoside triphosphates and were elongated for 90 min. The result of this experiment indicated that plasmid DNA containing the ML or the Eiv promoters (containing the TATA sequence) inhibited transcription from

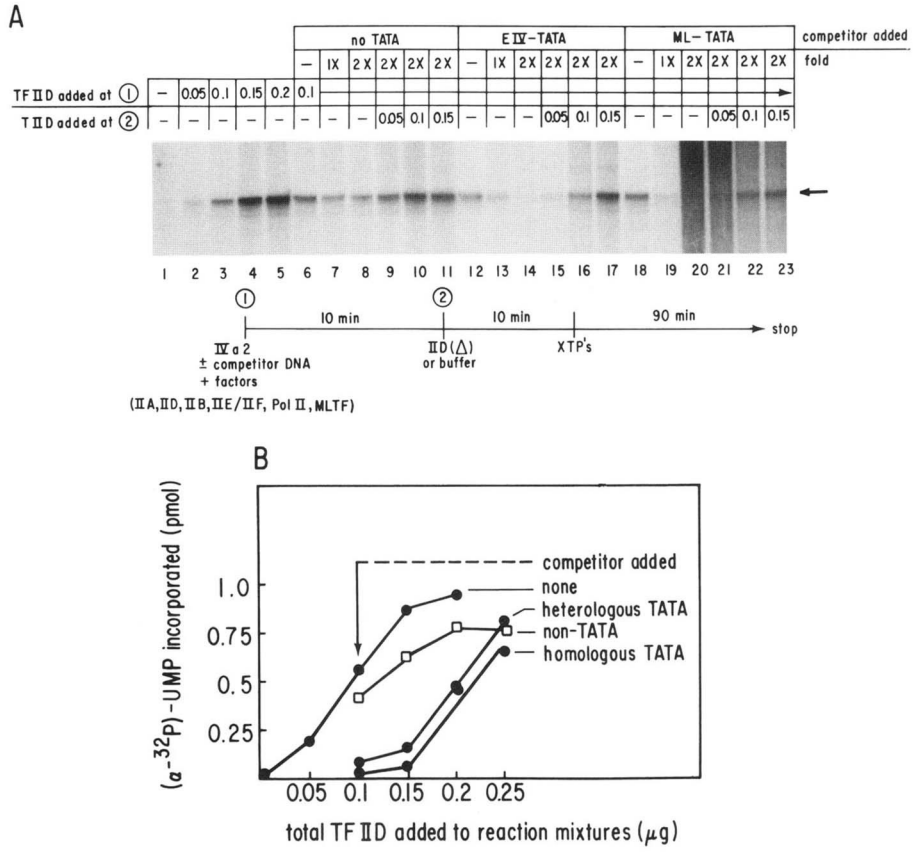

FIG. 8. **Transcription from the IVa2 promoter requires TFIID.** *Panel A,* reaction mixtures containing the general transcription factors IIA, IIB, IIE/IIF, RNA polymerase II, MLTF, and different amounts of TFIID (as indicated in the figure under ①) and DNA (the IVa2 promoter (pAd-204, 10 μg/ml, lanes 1–23) and, in addition, the Eiv promoter (pE4Δ-227/-11 (*lanes 8–11*) or pE4Δ-227/-46 (*lanes 13–17*) or the Ad-MLP (pD-139 (*lanes 20–23*), as indicated at the top of the figure by *1X* (10 μg/ml) or *2X* (20 μg/ml), were incubated as described under "Materials and Methods." After 10 min at 30 °C, some tubes (*lanes 9, 10, 11, 15, 16, 17,, 21, 22,* and *23*) received an additional amount of TFIID, as indicated on the figure under ②). After another 10 min of incubation, mixtures were supplemented with nucleotides and elongated for 90 min. Products of the reactions were separated on a 4% polyacrylamide-urea gel as described. *Panel B,* quantitative analysis of the data presented in *panel A.* Bands of the gel corresponding to transcription from the IVa2 promoter which are indicated by an *arrow,* were excised and counted. The amount of [α-^{32}P]UMP incorporated into the specific transcript was determined and plotted as a function of the TFIID concentration added to the reaction. The *arrow* denotes the concentration of TFIID at which a second template was added.

the IVA2 promoter (*lanes 19, 20* and *13, 14,* respectively, also see Fig. 8*B*). However, the addition of a plasmid DNA containing the Eiv promoter but lacking the TATA sequence had no or little effect on transcription from the TATA-less promoter (Fig. 8*A*, *lanes 7* and *8*, also see Fig. 8*B*). The inhibition observed by the ML and Eiv promoters could be overcome by the addition of TFIID (Fig. 8, *A* and *B*). The results of this experiment strongly suggest that TFIID was required for transcription from the IVa2 promoter and that this requirement was not due to a contaminant present in that protein fraction, but rather to a direct effect of TFIID on transcription from the IVa2 promoter.

In order to further analyze the effect of MLTF in transcription from the TATA-less promoter, point mutations that affected the binding of MLTF to its cognate site were introduced into the DNA template (see Fig. 11*A*). The transcriptional template activity of these mutants was analyzed in a IVa2 transcription system reconstituted with partially purified factors. Point mutations (T-G) at position −156 (−54 relative to the MLP CAP site) or at position −154 (A-C), resulted in an inhibition of the binding of MLTF to the promoter (Fig. 11*C*). MLTF binding correlated directly with the level of transcription from the IVa2 promoter. The point mutation at position −156, which resulted in a decrease of the binding of MLTF to its site, also resulted in a decrease of the levels of transcription; however, transcription from an internal control, the Eiv promoter, was not affected (Fig. 11*B*, compare *lanes 1* and *2*). Furthermore, a mutation (position −154, A-C) that almost abolished the binding of MLTF to its cognate site (Fig. 11*C*, compare *lanes 2* and *3* with *6* and *7*), yielded only basal levels of transcription from the IVa2 promoter (Fig. 11*B*, *lane 3*).

The above results strongly suggested that TFIID participated in transcription from the TATA-less promoter and also demonstrated that MLTF was required for maximal levels of transcription from the IVa2 promoter. The MLTF-mediated stimulation required TFIID.

DISCUSSION

Our studies indicated that transcription from the ML and IVa2 promoters was regulated by a common DNA element, the MLTF recognition site.

The purification of MLTF resulted in the isolation of two major polypeptides, one of 46,000 and another of approxi-

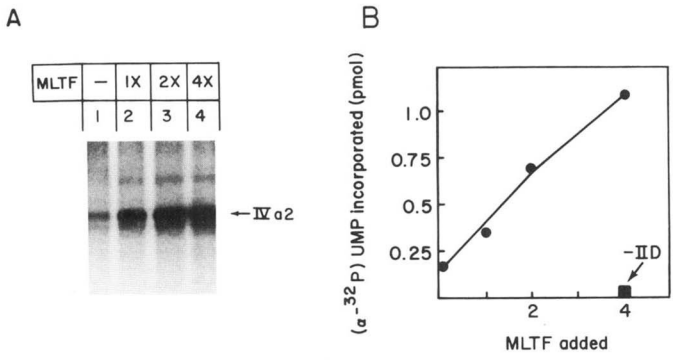

FIG. 9. **The effect of MLTF on transcription from the IVa2 promoter.** Reaction mixtures were as described under "Materials and Methods" and different amounts of MLTF were added as indicated in the figure (*panel A*). Products of the reactions were analyzed as described and the amount of [α-^{32}P]UMP incorporated into the specific transcript was determined by excising the bands from the dry gel and counting. The extent of transcription from the IVa2 promoter with respect to the amount of MLTF added to the reaction is presented in *panel B*. The amount of transcription observed in the absence of TFIID, which is indicated in *panel B*, was derived from the experiment presented in Fig. 8 (*lane 1*).

mately 50,000 daltons. The 46,000-dalton protein appears to coelute with the specific DNA binding activity (Fig. 1). This is in agreement with the studies of Chodosh *et al.* (1986), which demonstrated that the DNA binding activity of MLTF was contained in a polypeptide of 46,000 daltons. Interestingly, the studies of Moncollin *et al.* (1986), resulted in the isolation of a protein of approximately 55,000 daltons. This protein cosedimented on a glycerol gradient with both, DNA binding and transcription activities. It is possible that the transcription and DNA binding activities are contained in the 50,000-dalton polypeptide which we detected in our preparations of MLTF and that the 46,000 daltons is a proteolyzed form of a larger protein. It is important to mention that many of our preparations of MLTF resulted in the isolation of a protein which was devoid of transcription stimulatory activity but retained the DNA binding activity. Furthermore, the studies of Safer *et al.* (1988), resulted in the isolation of a 116,000-dalton protein that specifically recognized the MLTF recognition site. The relationship of the 116,000-dalton polypeptide with the other two proteins is unknown.

Using a highly purified preparation of MLTF and a functional transcription assay reconstituted with purified factors, we have analyzed the mechanism by which MLTF stimulated transcription from the MLP. Our studies indicated that the addition of MLTF to a transcription system reconstituted with the general transcription factors resulted in approximately 10-fold stimulation of transcription from the MLP. The stimulation appears to be the result of an increase in the stability of the preinitiation complex. Reactions carried out in the presence of MLTF increased the amount of complex formed by 2-fold (Figs. 3 and 5). Furthermore, preinitiation complexes formed at the MLP which contained the MLTF were more resistant to challenge by a DNA that contained the MLP than complexes formed in the absence of MLTF (Fig. 4).

The direct effect of MLTF was to produce a stable committed complex (defined by the formation of a DNA-protein complex which confers resistance of the transcription reaction to low concentrations of Sarkosyl (Hawley and Roeder, 1985)). It was previously demonstrated that the committed complex, containing TFIIA and TFIID, served as an entry site for RNA polymerase II into the transcription cycle (Fire *et al.*, 1984a; Reinberg *et al.*, 1987a). The studies described here indicated that MLTF was required for the formation of a Sarkosyl-resistant complex; however, the MLTF requirement could be partially overcome by RNA polymerase II (Fig. 5). These results suggested that MLTF and/or RNA polymerase II could stabilize a preformed complex containing TFIID and TFIIA. These results are consistent with the studies presented here which indicated that the DNA templates in reactions carried out in the presence of MLTF undergo multiple rounds of transcription. Reactions carried out in the absence of MLTF formed a stable committed complex only when the polymerase was part of it; thus when the polymerase leaves to undergo elongation, the complex dissociates. However, reactions carried out in the presence of MLTF formed a stable committed complex independent of the polymerase, thus when the polymerase translocated during elongation, the complex remained at the promoter and served as recognition for a second RNA polymerase II molecule. Our results are in agreement with the studies of Sawadogo and Roeder (1985a),

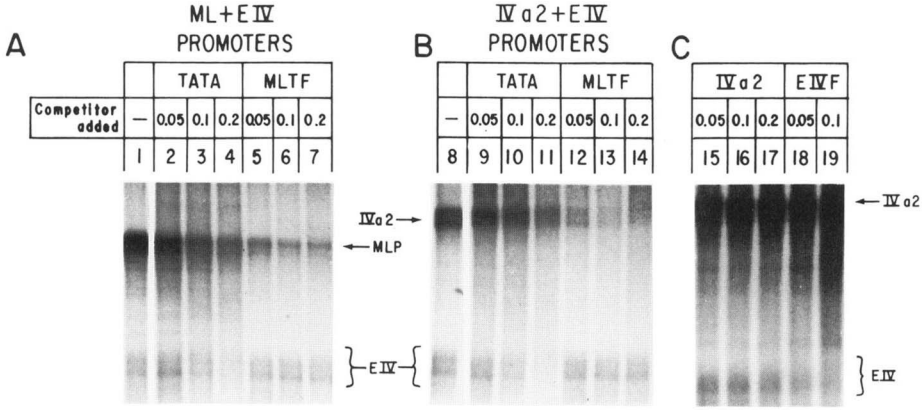

FIG. 10. **Oligonucleotides containing the MLTF or TFIID recognition site inhibited transcription from the IVa2 and ML promoters.** Transcription from the ML (*lanes 1–7*) and the IVa2 (*lanes 8–19*) promoters was reconstituted with partially purified general transcription factors and MLTF, as described under "Materials and Methods." A plasmid DNA containing the Eiv promoter and EivF (Sephacryl S-200 fraction, 0.3 μg, Cortes *et al.*, 1988) was also added to the different transcription mixtures. In addition different amounts of oligonucleotide containing the recognition site for MLTF (Ad-MLP sequences from −73 to −48), the TATA binding protein of the MLP (Ad-MLP sequences from −46 to −21), EivF (see Cortes *et al.*, 1988) or no known factor (IVa2), were added as indicated. Reactions were incubated at 30 °C for 45 min. Products were separated by electrophoresis on a 4% polyacrylamide-urea gel. *Lanes 1* and *8* represent reactions that received no oligonucleotide.

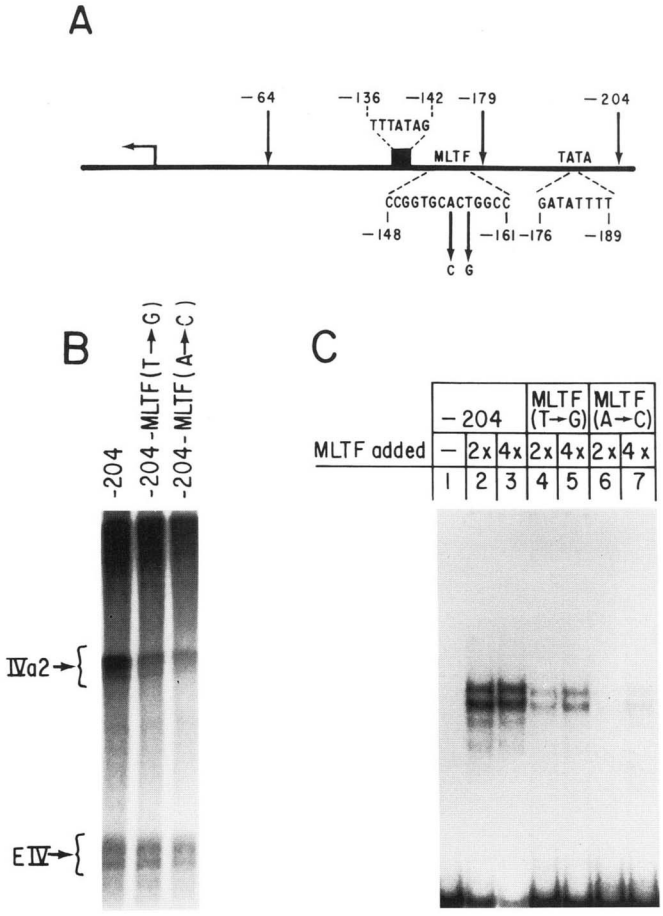

FIG. 11. **Inhibition of transcription from the IVa2 promoter by mutations in the MLTF recognition site.** *Panel A,* schematic representation of the IVa2 promoter. The MLTF recognition site and two point mutations are illustrated. Also the location of the MLP-TATA box is indicated. *Panel B,* transcription template activity of different IVa2 promoter constructs. Reactions were as described under "Materials and Methods" and contained the general transcription factors, RNA polymerase II, MLTF, and plasmid DNAs containing the IVa2 promoter with different mutations as indicated in the figure. In addition, reactions also received a plasmid DNA containing the Eiv promoter (4 μg/ml) and EivF (Sephacryl S-200 fraction, 0.14 μg (Cortes *et al.*, 1988)). Products of the reactions were analyzed as described. *Panel C,* analysis of the MLTF DNA binding activity to the wild type and two mutant DNAs. Reaction conditions and separation of the DNA-protein complex by electrophoresis on a 4% polyacrylamide gel were as described under "Materials and Methods." The amounts of MLTF added and the DNA used in this analysis are indicated in the figure.

that demonstrated that the addition of a partially purified protein fraction containing the major late upstream promoter factor, to a DNase I footprinting reaction that studied the binding of the TATA binding protein to its cognate site, resulted in the formation of a more stable DNA protein complex. Furthermore, they also postulated that the upstream factor could permit DNA template molecules to undergo multiple rounds of transcription.

The Ad-MLP CAP site is separated by 210 nucleotides from the CAP site of the IVa2 promoter, a non-TATA sequence containing promoter which is transcribed from the opposite DNA strand (Baker *et al.*, 1979; Baker and Ziff, 1981). Two DNA elements have been defined as positively regulating this promoter; a G/C-rich proximal element centered at position −40, relative to the IVa2 CAP site, and a distal element that overlaps with the MLTF binding site (Natarajan *et al.*, 1984, 1985, 1987). The studies of Lennard and Egly (1987), indicated that the MLTF recognition site could function bidirectionally, as inversion of the site had no effect on transcription from the MLP *in vivo* and *in vitro*. The studies presented here demonstrated that MLTF directly stimulated transcription from the IVa2 promoter (Figs. 8, 10, and 11). The stimulation was dependent on the MLTF recognition site (centered at position −155, relative to the IVa2 CAP site), as point mutations that affected the binding of MLTF directly affected the levels of transcription from the IVa2 promoter *in vitro*. This result suggests that MLTF can regulate transcription from both the IVa2 and ML promoters and further demonstrated that this site can function independently of its orientation, consistent with the dyad-symmetry sequence of the MLTF recognition site. Unfortunately, because sequences in and around the MLP-CAP site have an inhibitory effect on transcription from the IVa2 promoter (Natarajan *et al.*, 1985), we were unable to analyze the MLTF-mediated stimulation of both promoters simultaneously.

The IVa2 and ML promoters are both expressed late in the adenovirus infection cycle. The studies of Lennard and Egly (1987), indicated that the MLTF binding site served as recognition for a monomeric MLTF molecule. It is puzzling at this time how the binding of one MLTF molecule can simultaneously regulate transcription from both promoters. In our efforts to address this question, we have reconstituted transcription from the IVa2 promoter using partially purified factors. These studies indicated that transcription from the TATA-less promoter *in vitro*, was dependent on all the previously described general transcription factors, including TFIID, the TATA binding protein. The analysis of the DNA sequence that affected transcription from this promoter indicated that the MLP-TATA sequence plays no role or a negative effect in transcription from the IVa2 promoter (Natarajan *et al.*, 1984, 1985). However, the requirement of TFIID was demonstrated by reconstitution experiments and by oligonucleotide competition experiments. This analysis indicated that plasmid DNAs containing a TATA box element inhibited transcription from the IVa2 promoter (Fig. 9); however, the inhibition could be overcome by the addition of TFIID. The competition experiments argue against the possibility of a contaminant in the TFIID protein fraction that is required for transcription from the IVa2 promoter. However, these experiments do not rule out the possibility that the TATA binding proteins is in a complex with another factor which is required for transcription from the IVa2 promoter. It is possible that TFIID can recognize different DNA sequences, other than the MLP-TATA box, or that the DNA sequence specificity of TFIID can be modulated by a factor(s) that interacts with TFIID. Certainly the role of the TFIID protein fraction in transcription from the IVa2 promoter remains to be investigated further.

The experiments described here indicated that TFIID was required for transcription of the IVa2 promoter and that MLTF can modulate expression of two physically linked promoters; the MLP and the IVa2 promoter, a non-TATA sequence containing promoter.

Acknowledgments—We wish to thank Drs. K. J. Marians, M. Inouye, and N. Stone for reading the manuscript; O. Flores and P. Cortes from our laboratory for providing us with purified general transcription factors and EivF, respectively; Drs. M. Sawadogo and R. G. Roeder for providing the plasmid DNA pML(C2AT). The expertise of Dr. Nai-Sheng Lin in preparing the nuclear extracts is also acknowledged. We also thank the members of the laboratory and Dr. L. D. Vales for active discussions.

REFERENCES

Baker, C. C., and Ziff, E. B. (1981) *J. Mol. Biol.* **149,** 189–221

Baker, C. C., Herisse, J., Courtois, G., Galibert, F., and Ziff, E. (1979) *Cell* **18,** 569–580

Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* **50,** 349–383

Carthew, R. W., Chodosh, L. A., and Sharp, P. A. (1985) *Cell* **43,** 439–446

Carthew, R. W., Chodosh, L. A., and Sharp, P. A. (1987) *Genes Dev.* **1,** 973–980

Chodosh, L. A., Carthew, R. W., and Sharp, P. A. (1986) *Mol. Cell. Biol.* **6,** 4723–4733

Chodosh, L. A., Carthew, R. W., Morgan, J. G., Crabtree, G. R., and Sharp, P. A. (1987) *Science* **238,** 684–687

Cohen, R. B., Yang, L., Thompson, J. A., and Safer, B. (1988) *J. Biol. Chem.* **263,** 10377–10385

Concino, M. F., Lee, R. F., Merryweather, J. P., and Weinmann, R. (1984) *Nucleic Acids Res.* **12,** 7423–7433

Cortes, P., Leza, A., Rak, N., Hearing, P., Merino, A., and Reinberg, D. (1988) *Genes Dev.* **2,** 975–990

Davidson, B. L., Egly, J. M., Mulvihill, E. R., and Chambon, P. (1983) *Nature* **301,** 680–686

Dignam, D. J., Lebowitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1485

Dorn, A., Bollekens, J., Staub, A., Benoist, C., and Mathis, D. (1987) *Cell* **50,** 863–872

Dynan, W. S., and Tjian, R. (1983a) *Cell* **32,** 669–680

Dynan, W. S., and Tjian, R. (1983b) *Cell* **35,** 79–87

Dynan, W. S., and Tjian, R. (1985) *Nature* **316,** 774–778

Fire, A., Samuels, M., and Sharp, P. A. (1984) *J. Biol. Chem.* **259,** 2509–2516

Flores, O., Maldonado, E., Burton, Z., Greenblatt, J., and Reinberg, D. (1988) *J. Biol. Chem.* **263,** 10812–10816

Hawley, D. K., and Roeder, R. G. (1985) *J. Biol. Chem.* **260,** 8163–8172

Hen, R., Sassone-Corsi, P., Corden, J., Gaub, M. P., and Chambon, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7132–7136

Jansen-Durr, P., Bouef, H., and Kedinger, C. (1988) *Nucleic Acids Res.* **16,** 3771–3786

Jones, N. C., Rigby, P. W. J., and Ziff, E. B. (1988) *Genes Dev.*, in press

Kadonaga, J. T., and Tjian, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5889–5893

Khoury, G., and Gruss, P. (1983) *Cell* **33,** 313–314

Lee, W., Mitchell, P., and Tjian, R. (1987) *Cell* **49,** 741–752

Lennard, A. C., and Egly, J. M. (1987) *EMBO J.* **6,** 3027–3034

Leza, A., and Hearing, P. (1988) *J. Virol.* **62,** 3003–3013

Lin, Y-S., and Green, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3396–3400

Mansour, S. L., Grodzicker, T., and Tjian, R. (1986) *Mol. Cell. Biol.* **6,** 2684–2694

McKnight, S., and Tjian, R. (1986) *Cell* **46,** 795–805

Miyamoto, N. G., Moncollin, V., Wintzerith, M., Hen, R., Egly, J. M., and Chambon, P. (1984) *Nucleic Acids Res.* **12,** 8779–8799

Moncollin, V., Miyamoto, N. G., Zheng, X. M., and Egly, J. M. (1986) *EMBO. J.* **5,** 2577–2584

Morgan, J. G., Courtois, G., Fourel, G., Chodosh, L. A., Campbell, L., Evans, E., and Crabtree, G. R. (1988) *Mol. Cell. Biol.* **8,** 2628–2637

Nasmyth, K., Stillman, D., and Kipling, D. (1987) *Cell* **48,** 579–587

Natarajan, V., Madden, M. J., and Salzman, N. P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6290–6294

Natarajan, V., Madden, M. J., and Salzman, N. P. (1985) *J. Virol.* **55,** 10–15

Natarajan, V., Madden, M. J., and Salzman, N. P. (1987) *J. Virol.* **61,** 646–652

Reinberg, D., and Roeder, R. G. (1987) *J. Biol. Chem.* **262,** 3310–3321

Reinberg, D., Horikoshi, M., and Roeder, R. G. (1987a) *J. Biol. Chem.* **262,** 3322–3330

Reinberg, D., Flores, O., and Buckbinder, L. (1987b) in *Molecular Biology of RNA: New Perspectives* (Inouye, M., and Dudock, B., eds) pp. 423–439, Academic Press, Inc., San Diego, CA

Safer, B., Cohen, R. B., Garfinkel, S., and Thompson, J. A. (1988) *Mol. Cell. Biol.* **8,** 105–113

Sawadogo, M., and Roeder, R. G. (1985a) *Cell* **43,** 165–175

Sawadogo, M., and Roeder, R. G. (1985b) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4394–4398

Weil, A. P., Luse, D. S., Segall, J., and Roeder, R. G. (1979) *Cell* **18,** 469–484

Yu, Y. T., and Manley, J. L. (1984) *Nucleic Acids Res.* **12,** 9309–9321