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Role of the Mammalian Transcription Factors IIF, IIS, and IIX during Elongation by RNA Polymerase II

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We have used a recently developed system that allows the isolation of complexes competent for RNA polymerase II elongation (E. Bengal, A. Goldring, and Y. Aloni, J. Biol. Chem. 264:18926–18932, 1989). Pulse-labeled transcription complexes were formed at the adenovirus major late promoter with use of HeLa cell extracts. Elongation-competent complexes were purified from most of the proteins present in the extract, as well as from loosely bound elongation factors, by high-salt gel filtration chromatography. We found that under these conditions the nascent RNA was displaced from the DNA during elongation. These column-purified complexes were used to analyze the activities of different transcription factors during elongation by RNA polymerase II. We found that transcription factor IIS (TFIIS), TFIIF, and TFIIX affected the efficiency of elongation through the adenovirus major late promoter attenuation site and a synthetic attenuation site composed of eight T residues. These factors have distinct activities that depend on whether they are added before RNA polymerase has reached the attenuation site or at the time when the polymerase is pausing at the attenuation site. TFIIS was found to have antiattenuation activity, while TFIIF and TFIIX stimulated the rate of elongation. In comparison with TFIIF, TFIIS is loosely bound to the elongation complex. We also found that the activities of the factors are dependent on the nature of the attenuator. These results indicate that at least three factors play a major role during elongation by RNA polymerase II.

RNA polymerase II sequentially utilizes several accessory factors during the multistage process of transcription (27). These factors can be functionally classified into three groups: (i) regulatory factors, which either directly bind to specific DNA sequences (18, 26) or regulate the activity of specific DNA binding proteins (18); (ii) general transcription factors, which together with RNA polymerase II constitute the basic transcription machinery required for basal levels of transcription (27, 44); and (iii) factors that affect elongation and termination by RNA polymerase II (21, 30, 34, 37, 50).

Recent studies have shown that the expression of several genes can be regulated by premature termination (1, 32, 41). Interestingly, it has been shown that premature termination during transcription of the mouse β-major globin gene (33), the c-*myc* gene (5), and the *Drosophila* heat shock gene *hsp-70* (43) can vary with the physiological state of the cells. Premature termination termed attenuation, has been shown to regulate the quantity of mRNA of several animal viruses, including simian virus 40 (SV40) (1, 14, 15, 48, 49), polyomavirus (47), the parvovirus minute virus of mice (2, 40), adenovirus type 2 (Ad2) (23, 24, 45), and human immunodeficiency virus types 1 and 2 (21, 54). These observations indicate that regulation of gene expression can be exerted at the level of elongation by RNA polymerase II. The findings that 3′-end formation of U1 and U2 small nuclear RNAs requires promoter elements (16, 29) and that a *cis*-acting element present in the promoter region of the murine c-*myc* gene is necessary for transcriptional block (28) represent important steps toward the understanding of this type of regulation. From these findings, it is tempting to hypothesize that factors regulating termination or antitermination can recognize, directly or indirectly, promoter elements and thereby affect the fate of the elongating polymerase.

An essential step toward understanding the factors regulating elongation at the molecular level is the development of in vitro systems that can mimic the in vivo observations. Three different approaches have been used to isolate activities that affect elongation by RNA polymerase II: (i) a promoter-independent assay that uses double-stranded DNA containing a 3′-end deoxycytidylic tail (31, 38, 50), (ii) a promoter-dependent assay reconstituted with purified transcription factors (37), and (iii) a promoter-dependent assay in which initiation of transcription and partial elongation take place in crude cell extracts. Elongation complexes are then purified by using gel filtration chromatography in high salt (4).

By using these assays, several factors affecting elongation by RNA polymerase II have been isolated. Factor SII, initially purified from mouse cells (46), which seems to be analogous to factor SII purified from calf thymus (34), to transcription factor IIS (TFIIS) purified from HeLa cells (37), and to factor DmS-II purified from *Drosophila* Kc cells (50), has been shown to bind to RNA polymerase II (17). The calf thymus-derived factor was found to interact with the largest subunit of RNA polymerase II (34). Human TFIIS and calf thymus SII were shown to be required for transcription elongation and to have no role in initiation (34, 37). However, TFIIF, a factor purified from HeLa cells (11), and factor 5, purified from *Drosophila* Kc cells (30, 31), are required for specific initiation of transcription (12, 30) and also affect elongation by RNA polymerase II (13, 31).

In this study we have used purified transcription complexes, which are depleted of elongation factors (4), and characterized the activities of different transcription factors during elongation by RNA polymerase II. Our results indicate that at least three transcription factors affect elongation.

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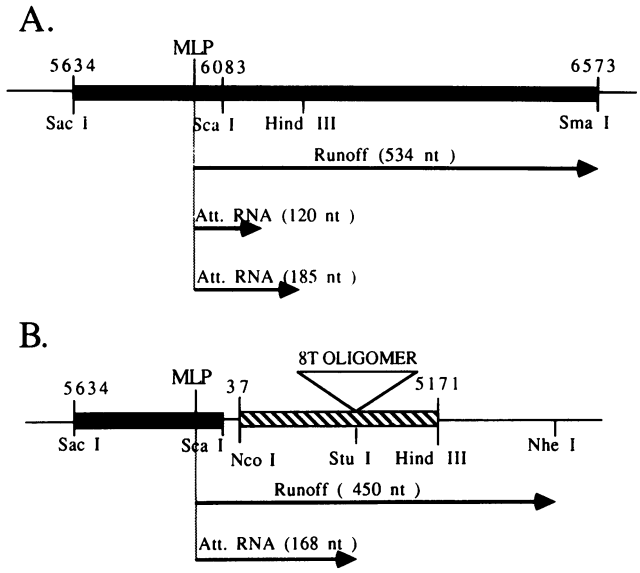

FIG. 1. Schematic representation of DNA templates. (A) Plasmid DNA pGEM-Ad2. This plasmid contains Ad2 MLP sequences (boxed regions) and was constructed as described in Materials and Methods. The DNA was digested with the endonuclease *Sma*I; transcription from the Ad2 MLP to the *Sma*I end generates RNA molecules of 534 nucleotides (nt) (runoff RNA). The lengths of the Ad2 attenuated RNAs (Att. RNA) resulting from pausing of the RNA polymerase II are also indicated. (B) A plasmid DNA containing a synthetic attenuator. This plasmid is composed of Ad2 DNA sequences containing the Ad2 MLP (■), linker sequences (——), and SV40 DNA sequences (▨) and was constructed as described in Materials and Methods (4). A 16-bp DNA fragment containing a stretch of eight T residues was inserted at the unique *Stu*I site present in the SV40 DNA fragment (▽). The DNA was digested with endonuclease *Nhe*I. Initiation at the MLP with elongation to the end of the DNA fragment generated transcripts of 450 nucleotides (runoff). The lengths of RNA molecules initiated at the Ad2 MLP which paused at the synthetic attenuation site (Att. RNA) are also indicated.

MATERIALS AND METHODS

DNA templates. A *Sac*I-*Sma*I Ad2 DNA fragment (5634 to 6573, Ad2 numbering) was inserted at the *Sac*I and *Sma*I sites present in the polylinker of the pGEM 1 DNA, creating the pGEM-Ad2 plasmid DNA. This plasmid DNA contained the Ad2 major late promoter (MLP) and was used for transcription in vitro. The DNA was digested with the *Sma*I endonuclease, and transcription from the MLP to the *Sma*I end generated an RNA product of 534 nucleotides (Fig. 1A). The construction of a plasmid DNA with a synthetic deoxynucleotide containing eight consecutive T residues downstream of the MLP and in the sense DNA strand has been previously described (3). This plasmid DNA contains 450 bp of Ad2 DNA (nucleotides 5634 to 6083), which were inserted in the polylinker of pGEM 1 DNA. This DNA molecule also contained SV40 DNA sequences (nucleotides 37 to 5171, generated by digestion with *Nco*I and *Hin*dIII; Fig. 1B). A chemically synthesized oligonucleotide containing eight consecutive T residues was inserted in the unique *Stu*I site present in the SV40 sequences. The resulting plasmid DNA was digested with endonuclease *Nhe*I and used for transcription in vitro. Transcription starting at the Ad2 MLP and extending to the *Nhe*I site generated an RNA molecule of 450 nucleotides (Fig. 1B).

Transcription in vitro and isolation of elongation complexes. Whole cell extract was prepared as described previously (25). Reaction mixtures (160 μl) containing 40 μl of whole cell extract (20 mg/ml) and plasmid DNA carrying the Ad2 MLP (8 μg) were incubated in a buffer containing 50 mM KCl, 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (vol/vol) glycerol, 4 mM creatine phosphate, and 1 mM dithiothreitol (DTT) (preinitiation step). After 30 min at 30°C, 50 μM ATP and 240 μCi each of [α-^{32}P]CTP and [α-^{32}P]UTP were added, and the mixture was incubated for 3 min. The sample was then applied directly onto a Sephacryl S-1000 column (4-ml bed volume) equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 300 mM KCl. Fractions of 60 μl were collected, and radioactivity was determined. The void volume of the column was 2.5 ml, and the first fraction that contained radioactivity was taken as fraction 1. Each fraction was then diluted to 360 μl with elongation chase buffer (20 mM Tris-HCl [pH 7.9], 5 mM $MgCl_2$, 4 mM creatine phosphate, 1 mM DTT, 700 μM each of the four ribonucleoside triphosphates), bringing the KCl concentration to 50 mM. Where indicated, a few fractions containing transcription complexes were pooled. The pooled fractions were then divided into several fractions to which the elongation chase buffer was added with or without the indicated transcription factors. Elongation was allowed to proceed for 20 min at 30°C. The elongation reaction was stopped by the addition of 20 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 μg of proteinase K per ml, and 30 μg of tRNA, and samples were incubated at 37°C for 10 min. RNA was phenol-chloroform extracted, purified by centrifugation through a G-25 minicolumn, and collected by ethanol precipitation and centrifugation. RNA was resuspended in 5 to 10 μl with 90% formamide and analyzed by electrophoresis on 6% polyacrylamide gels (bisacrylamide/acrylamide, 1:19) containing 7 M urea. Electrophoresis was carried out at a constant current of 20 mA.

RNase H assay. A labeled RNA probe was synthesized by using T7 RNA polymerase and pGEM 1 DNA template digested with the restriction endonuclease *Nae*I. Transcription by T7 RNA polymerase from this DNA template generated a 324-nucleotide RNA. Reaction conditions were as described by Promega. The labeled RNA was gel purified.

RNA produced during specific transcription by the isolated elongation complexes during a 20-min chase as described above was mixed with the T7 RNA probe and with an SP6 promoter DNA primer (Promega) (50 ng) complementary to sequences in both the T7 probe (which serves as an internal control) and the specific transcript. The primer should hybridize to the specific RNA only if it is displaced from the template DNA. This was analyzed by the addition of RNase H (2 U; Boehringer Mannheim). The mixture was then incubated for 60 min. The products of the RNase H reaction were analyzed by electrophoresis on a 6% polyacrylamide-urea gel.

Purification of transcription factors. Transcription factors were purified from HeLa cell nuclear extracts prepared as previously described (10). TFIIB was purified as described elsewhere (36). TFIIE was purified as described by Flores et al. (13). TFIIF was purified to near homogeneity as previously described (11). TFIID was purified as described by Carcamo et al. (8). TFIIX was purified as described by Reinberg et al. (35). TFIIS was a gift of R. Weinmann and was purified from calf thymus as described previously (34).

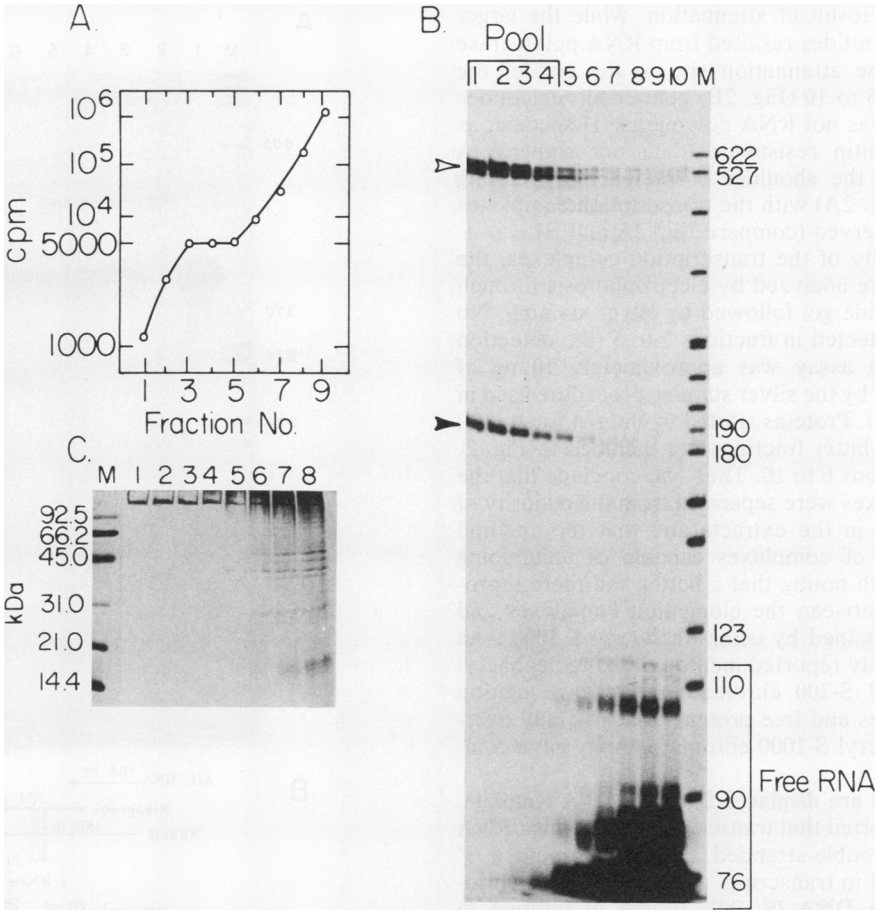

FIG. 2. Purification of elongation-competent complexes. Briefly elongated complexes were loaded onto a Sephacryl S-1000 column (see Materials and Methods). Fractions were collected, and the elution of radiolabeled material was monitored by Cernekov counting. (A) Typical elution pattern of radioactive material. (B) Transcription elongation. Elongation chase buffer was added to each fraction, and transcription elongation was allowed to proceed for 20 min. Reactions were stopped, and the RNA products were analyzed by electrophoresis as described in Materials and Methods. The attenuated RNA is indicated by a filled arrowhead, and the runoff transcript is marked by an open arrowhead. Fraction numbers are indicated at the top. Lane M, DNA size markers (indicated in nucleotides at the right). (C) Assay in which an aliquot (60 μl; the same amount used for transcription elongation) of each column fraction was analyzed by 12% SDS-polyacrylamide gel electrophoresis followed by silver staining. Fraction numbers are indicated at the top. The fraction numbers are the same in the three panels. Lane M, Protein molecular size standards.

RESULTS

Isolation of elongation-competent complexes. We have recently reported the development of a system that allows the analysis of activities that affect elongation by RNA polymerase II (4). Using this system, we carried out analyses on elongation-competent complexes generated after accurate initiation. Preinitiation complexes were formed at the Ad2 MLP and then briefly elongated in the presence of ATP and limiting amounts of labeled CTP and UTP but in the absence of GTP. This allows the preinitiation complex to start transcription and elongation to proceed for 10 nucleotides, where the elongating machinery encounters the first cytidylic residue and halts because of the absence of GTP. These elongation complexes were separated from proteins present in the extract, as well as from proteins loosely bound to the elongation complex and to the DNA template, by gel filtration chromatography through a Sephacryl S-1000 column in the presence of high salt (0.3 M KCl). The isolated complexes could be further elongated after reduction of the KCl concentration and addition of ribonucleoside triphosphates. We used this procedure to analyze the effect of different transcription factors during elongation by RNA polymerase II.

Previous studies have demonstrated the existence of two sites in the adenovirus major late transcriptional unit that can block elongation by RNA polymerase II in vivo (24, 45) and in vitro (23, 37). These sites are located 120 and 185 nucleotides downstream of the transcriptional initiation site. The site located at +185 is the stronger of the two (24, 37). Transcription complexes were formed at the Ad2 MLP and separated by chromatography as described in Materials and Methods. Complexes capable of undergoing elongation eluted from the gel filtration column in the first four fractions, as indicated by the elution pattern of radioactive material (Fig. 2A). Two discrete RNA molecules of approximately 185 and 540 nucleotides were synthesized after the fractions were elongated under chase conditions (Fig. 2B). These RNA molecules were produced only after incubation of the isolated complexes under elongation-chase conditions; no products could be detected when the isolated complexes were directly analyzed on polyacrylamide-urea gels (data not shown; see Fig. 6). The shorter RNA of 185

nucleotides was the result of attenuation, while the larger molecule of 534 nucleotides resulted from RNA polymerase II readthrough of the attenuation site to the end of the template. Fractions 6 to 10 (Fig. 2B) contained nucleotides and free RNA that was not RNA polymerase II specific, as detected by α-amanitin resistance (data not shown). A correlation between the shoulder of radioactivity eluting from the column (Fig. 2A) with the transcription complexes was consistently observed (compare Fig. 2A and B).

To assess the purity of the transcription complexes, the column fractions were analyzed by electrophoresis through an SDS-polyacrylamide gel followed by silver staining. No proteins could be detected in fractions 1 to 5 (the detection limit of the staining assay was approximately 10 ng of protein), as indicated by the silver staining procedure used in this analysis (Fig. 2C). Proteins started to elute in fraction 6, with most eluting in latter fractions not included in Fig. 2. RNA eluted in fractions 6 to 10. Thus, we conclude that the transcription complexes were separated from the majority of the proteins present in the extracts and that this method allows the isolation of complexes capable of undergoing elongation. It is worth noting that a better and more reproducible separation between the elongation complexes and free proteins was obtained by using Sephacryl S-1000 than by using the previously reported method utilizing Sephacryl S-200 (4). Sephacryl S-200 chromatography gave elution profiles for complexes and free proteins that partially overlapped, while Sephacryl S-1000 chromatography gave complete separation.

Nascent transcripts are displaced from the DNA template. Previous studies reported that transcription by purified RNA polymerase II on double-stranded DNA containing a 3′ poly(dC) tail resulted in transcripts which remained hybridized to the template DNA (9, 20). It was of interest to analyze whether the same unusual phenomenon occurs when transcription is dependent on a promoter and auxiliary proteins. This could be meaningful for regulation of transcription elongation.

Transcript displacement was determined by its sensitivity to RNase H digestion in the presence of a short complementary oligonucleotide and by RNase A digestion. Transcription-competent complexes were assembled and purified as described above except that a plasmid DNA that carries the Ad2 MLP followed by a synthetic attenuator of eight consecutive T residues on the coding strand was used as the template (see Materials and Methods and Fig. 1). Transcription from this DNA resulted in the production of RNAs of 450 (runoff) and 168 (attenuated) nucleotides (Fig. 1B).

Transcription complexes were isolated as described for Fig. 2A and elongated for 20 min as described in Materials and Methods. To analyze whether the nascent RNA molecules were displaced from the template DNA, RNase H together with a 19-nucleotide primer DNA and a labeled T7 RNA was added, and the mixture was incubated for an additional 60 min. The primer DNA was complementary to both the specific runoff RNA and the T7 transcript but not to the attenuated RNA (Fig. 3B). If the runoff RNA is released from the DNA template, the DNA primer should hybridize to it, generating a specific substrate for RNase H. Figure 3B indicates the sites at which the DNA primer is complementary to the runoff RNA as well as to the control labeled T7 RNA and the expected sizes of the RNase H digestion products. The 3′ fragment of the digested runoff RNA was not labeled and therefore not shown in the diagram in Fig. 3B. RNase H specifically cleaved the runoff transcript (Fig. 3A, lane 7, arrow A) to produce a truncated transcript of the

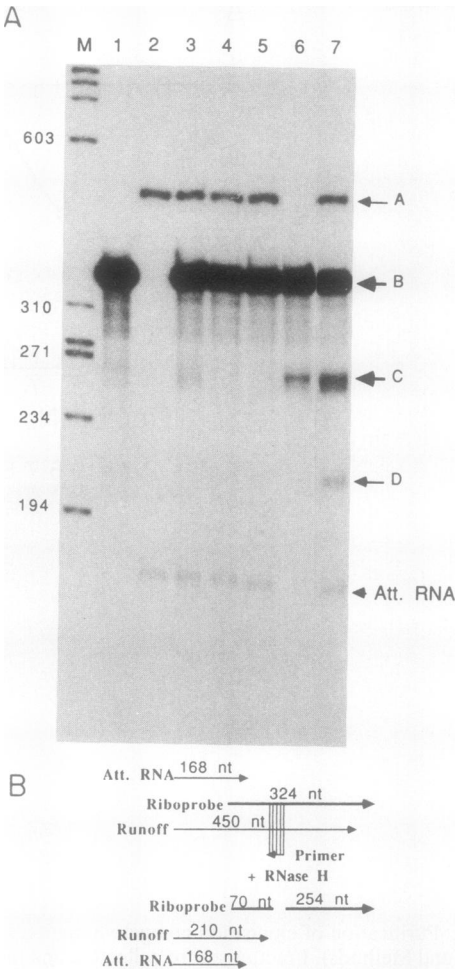

FIG. 3. Displacement of transcripts from the DNA template. (A) Transcription elongation. Transcription initiation from the synthetic template, purification of the briefly initiated transcription complexes, and transcription elongation were performed as described in Materials and Methods. After 20 min of elongation, the following components were added to individual reactions (lanes 2 to 5 and 7) and incubation was allowed to continue for an additional 60 min. Lanes: M, size markers (indicated in nucleotides at the left); 2, no addition; 3, T7 riboprobe and DNA primer; 4, T7 riboprobe and RNase H; 5, T7 riboprobe; 7, riboprobe, RNase H, and DNA primer. Lane 1 shows the T7 riboprobe; lane 6 shows the riboprobe incubated for 60 min in the presence of the primer and RNase H. Arrows A to D indicate the runoff transcript, undigested T7 riboprobe, RNase H digestion product of the riboprobe, and RNase H digestion product of the runoff RNA, respectively. Att. RNA, The 168-nucleotide attenuated RNA. (B) Schematic representation of the expected RNA products sizes before and after RNase H digestion.

expected size (210 nucleotides; arrow D), as well as the T7 transcript (arrow B) to produce a truncated 254 nucleotide RNA (arrow C). The efficiency of specific cleavage by RNase H in this experiment was measured by gel densitometry. It was found that 25 to 30% of the total transcripts were cleaved when the primer was annealed with either the control T7 RNA or the runoff transcript, indicating that the runoff RNA was displaced from the template. The apparent low cleavage efficiency results from the complexity of the assay. Both transcripts are probably folded in an extensive secondary structure that prevents effective hybridization of the DNA primer to the labeled RNA molecules. The alter-

native possibility, that only a small fraction of the transcripts was displaced from the DNA template, can be ruled out, since the efficiencies of RNase H digestion of the runoff RNA and the control T7 transcript were similar. Moreover, no digestion by RNase H could be observed in the absence of DNA primer from either the runoff or the control T7 RNA (Fig. 3A, lane 4, arrows A and B). Also, both the runoff and the attenuated RNAs were sensitive to RNase A digestion under conditions in which RNA-DNA hybrids are RNase A resistant (results not shown). This finding excludes the possibility that the attenuated and runoff RNAs were hybridized to the template. We therefore concluded that the purified transcription complexes were able to fully displace the transcripts from the DNA template.

Effect of the general transcription factors during elongation by RNA polymerase II. Previous studies have shown that five accessory protein factors (TFIIA, -IIB, -IID, -IIE, and -IIF), in addition to RNA polymerase II, are required for basal levels of transcription (27, 44). These studies have shown that these factors appear to be required for initiation of transcription of all class II genes; therefore, they have been defined as general transcription factors. In addition, factors affecting elongation or termination by RNA polymerase II have been described (21, 31, 34, 37, 38, 50). One factor, termed TFIIS (37) or SII (34, 46), has been shown to influence the ability of RNA polymerase II to read through specific pausing sites. Recent reports have suggested that the general transcription factor TFIIF (12), which is required for the formation of a stable preinitiation complex, also may affect elongation by RNA polymerase II (13). The analysis of TFIIF on elongation used a 3′-end poly(dC)-extended template in which the effect of the factors required for specific transcription initiation was bypassed (13). Therefore, we restudied the effects of the different transcription factors on elongation by RNA polymerase II by using a transcription assay that was dependent on a specific promoter.

The addition of highly purified preparations of TFIIS (34) or TFIIF (11) to isolated transcriptional complexes affected the efficiency of elongation, as indicated by the disappearance of the 185-nucleotide attenuated RNA, while the runoff transcripts increased proportionally as a function of factor concentration (Fig. 4; compare lane 1 with lanes 2 to 10). The effect of TFIIF during elongation was not due to a contaminating activity in the TFIIF fraction used, as antibodies directed against RAP 30 (a component of TFIIF [12]), which inhibited transcription initiation (data not shown; see references 6 and 12), also specifically inhibited the elongation-stimulatory activity (Fig. 5; compare lane 2 with lanes 3 to 5).

Interestingly, an effect similar to those of TFIIS and TFIIF was observed with TFIIX (Fig. 4; compare lane 1 with lanes 11 to 15), an activity initially identified as stimulating transcription from the Ad2 MLP when sequences downstream of +33 (relative to the MLP cap site) were present (35). The observed effect on elongation was specific for TFIIS, TFIIF, and TFIIX; the addition of TFIIA, TFIIB, TFIID, or TFIIE did not stimulate readthrough of the attenuation site (data not shown; see below). We were also unable to see any effect of these transcription factors when they were added in combination with a concentration of TFIIF or TFIIS capable of releasing approximately 50% of the RNA polymerase II molecules from the pausing site (data not shown).

TFIIF, TFIIS, and TFIIX have different antitermination activities. We indicated above that TFIIF, TFIIS, and TFIIX could prevent pausing at the Ad2 MLP attenuation site.

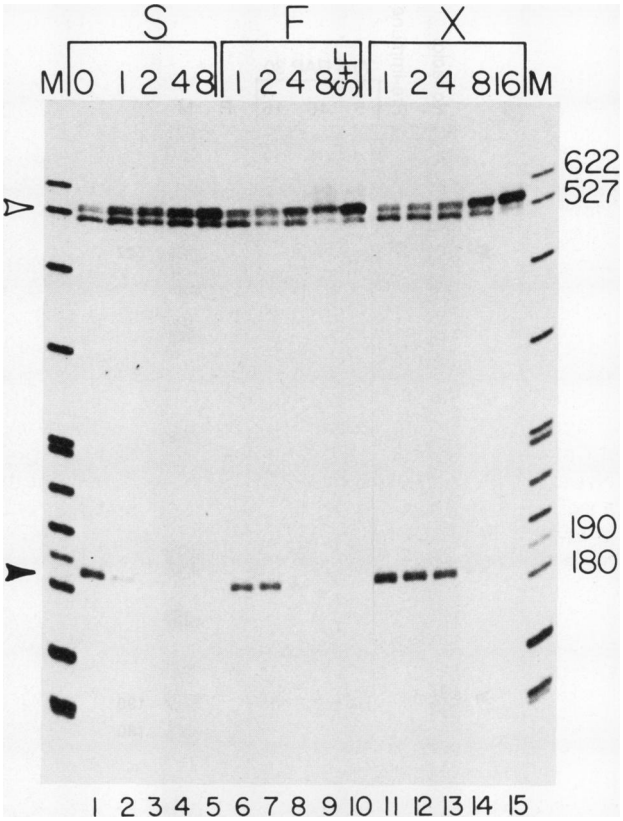

FIG. 4. Evidence that TFIIS, TFIIF, and TFIIX can prevent blockage of transcription elongation at the Ad2 185-nucleotide attenuation site. Transcription complexes were assembled and purified as described in Materials and Methods. TFIIF, TFIIS, and TFIIX (F, S, and X) were added at different concentrations, and elongation was allowed to proceed for 20 min. The protein concentrations of TFIIF and TFIIS could not been defined because the activities were purified to apparent homogeneity, and bovine serum albumin was added to preserve the transcriptional activity (for details, see references 11 and 25). Numbers at the top indicates volume (microliters) added. The open and filled arrowheads indicate the runoff RNA and the attenuated RNAs, respectively. M, DNA size markers (indicated in nucleotides at the right).

Under the conditions analyzed in Fig. 4, the factors were added at an early stage of elongation, before the polymerase had reached the attenuation site (see above and below). Next, we analyzed whether the factors would still be able to release the elongation block if the transcription complexes were allowed to reach the attenuation site.

Transcription complexes were isolated as described and then elongated with cold nucleotide in the absence of added factor (Fig. 6, lanes Chase 1 and Chase 2). After 20 or 40 min of chase, few transcription complexes were capable of passing through the attenuation site (lanes 6 and 11). The attenuated RNA (filled arrowhead) accumulated during the chase (compare lane 1 with lanes 6 and 11) and reached maximal levels after 5 min (data not shown; see below). In agreement with the results presented above (Fig. 4), the addition of TFIIF, TFIIS, or TFIIX together with the cold nucleotides (beginning of the chase) resulted in efficient readthrough of the attenuation site (compare lane 6 with lanes 2 to 5). Interestingly, a different situation was observed when TFIIF was added after the elongation complexes had reached the attenuation site. Under these conditions, TFIIF

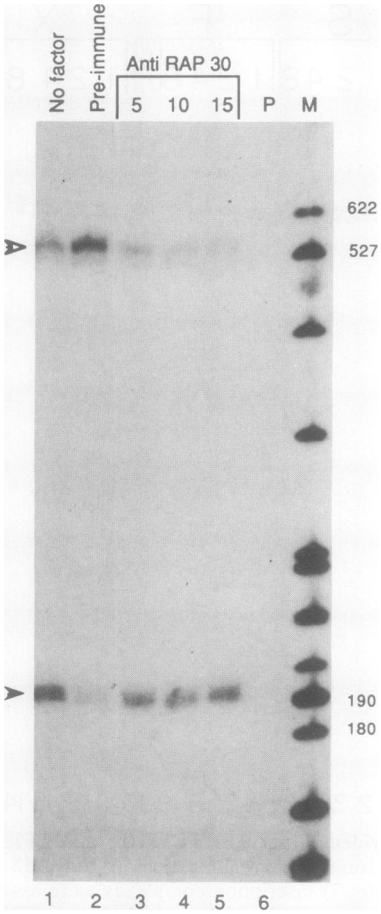

FIG. 5. Inhibition by anti-RAP 30 antibodies of TFIIF transcription elongation activity. Briefly initiated transcription complexes were formed and purified as detailed in Materials and Methods and the legend to Fig. 2. The supernatants obtained after immunoprecipitation of TFIIF with either preimmune serum (lane 2) or anti-RAP 30 antibodies (lanes 3 to 5) (amounts [microliters] of supernatant added are indicated at the top) were added to the purified complexes, and elongation was allowed to proceed for 20 min. Lane 6 contained only elongation-competent complexes (pulse). Lane 1 shows the RNA produced after 20 min when no factor was added. The open and filled arrowheads indicate the runoff and attenuated RNAs, respectively. Lane M, DNA size markers (indicated in nucleotides at the right). Immunoprecipitations were performed as described previously (12). The antibodies used were against a peptide (NH_2-Arg-Pro-Ala-Ala-Ser-Glu-Asn-Tyr-Met-Arg-Leu-Lys-Arg-Leu-Gln-Ile-Glu-Glu-Ser-Ser-Lys-COOH) derived from the sequence of RAP 30 (52).

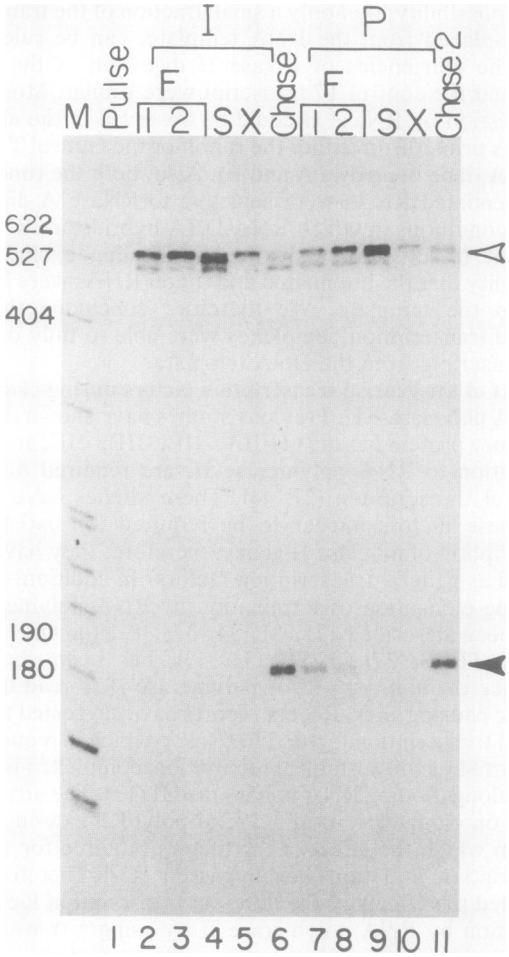

FIG. 6. Evidence that TFIIF cannot efficiently relieve elongation block when added to a paused polymerase. Pulse-labeled Ad2 transcription complexes were purified as described in Materials and Methods. TFIIF (F), TFIIS (S), and TFIIX (X) were added either with the elongation chase mixture and incubated for 20 min (lanes I) or after 20 min of elongation and incubated for an additional 20 min (lanes P). TFIIF was added at 4 and 8 μl (lanes 2 and 3). TFIIS and TFIIX were added at 2 and 8 μl, respectively. Other lanes: Pulse, column-purified complexes that were not chased; Chase 1 and Chase 2, transcription complexes that were chased in the absence of added factors for 20 and 40 min, respectively; M, DNA size markers (indicated in nucleotides at the left). The open arrowhead indicates the runoff RNA, and the filled arrowhead indicates the attenuated RNA. Note that a lower amount of radioactivity was loaded in lane 10. However, the same results were obtained in similar experiments in which higher amounts of RNA were loaded on the gel.

had a limited ability to release RNA polymerase II from the pausing site (compare lanes 7 and 8 with lanes 2 and 3). Since the same relative concentrations of factors were used when added at the beginning of the chase (lanes 2 to 5) or after the polymerase reached the pausing site (lanes 7 to 10), we concluded that TFIIF is less efficient than TFIIS and TFIIX in releasing the elongation block when the polymerase has already reached the attenuation site. Therefore, TFIIS did not distinguish between the two situations (the elongation complex prior to or at the attenuation site), while TFIIF did distinguish both situations. Quantitation of the amount of attenuated RNA accumulated with use of two different concentration of TFIIF indicated that only 26 and 50% of the RNA was released from the attenuation site (data not shown). To completely release the polymerase from the attenuation site, the concentration of TFIIF had to be increased fivefold (data not shown) with respect to the amount required to relieve pausing when added at the beginning of the chase (Fig. 6, lanes 2 and 3). On the other hand, TFIIS and TFIIX allowed the elongation machinery to traverse through the attenuation site independently of whether the polymerase was upstream of or at the attenuation site (compare lanes 4 and 5 with lanes 9 and 10).

TFIIF and TFIIX stimulate the rate of elongation by RNA polymerase II. To understand the effects of TFIIF, TFIIS, and TFIIX during elongation, kinetic studies were performed on column-purified transcription complexes in the presence and absence of the different transcription factors.

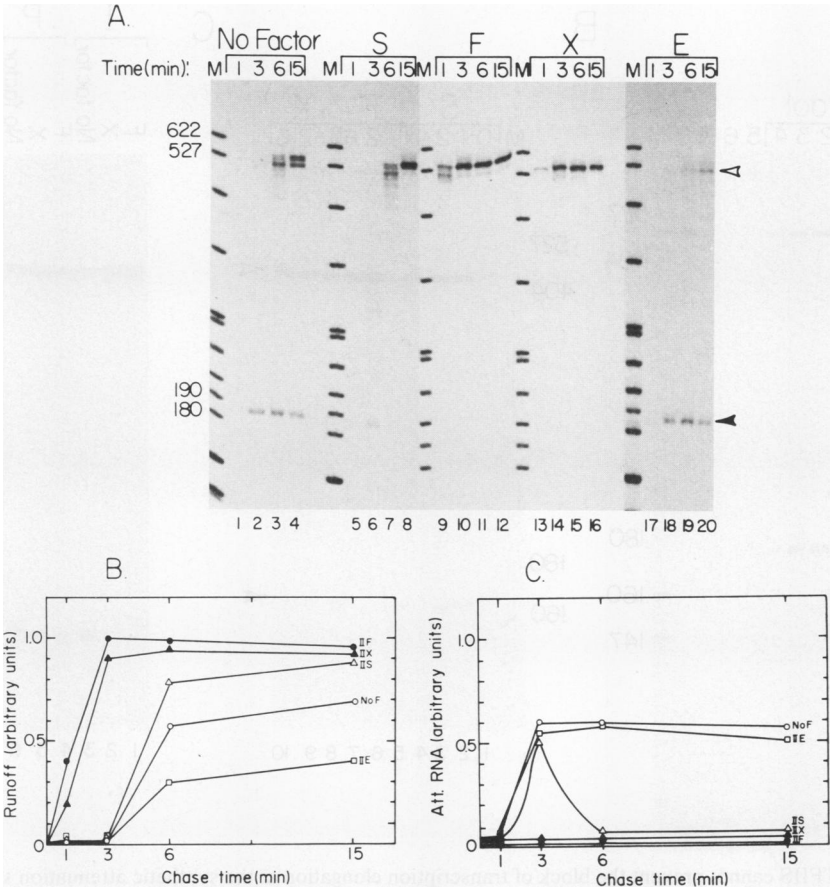

FIG. 7. Evidence that TFIIF and TFIIX but not TFIIS stimulate the rate of transcription elongation. (A) Electrophoretic analysis. Purified elongation-competent complexes were chased in the absence or presence of TFIIS, TFIIF, TFIIX, or TFIIE (S, F, X, or E) for the indicated times (1, 3, 6, and 15 min). The elongation factors were added at saturating amounts: TFIIF, 8 μl; TFIIS, 4 μl; and TFIIX, 16 μl. The reaction products were analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Lane M, DNA size markers (indicated in nucleotides at the left). (B and C) Quantitation of the levels of runoff and attenuated (Att.) RNAs synthesized in the absence of factors or in the presence of TFIIF, TFIIX, TFIIS, or TFIIE. Quantitation was performed by densitometric tracing of the autoradiogram.

When no factor was added to the elongating transcription complexes, the attenuated RNA was observed at 3 min (Fig. 7A and C) and the runoff was observed at 6 min (Fig. 7A and B). Surprisingly, the addition of TFIIS did not affect the initial rate of transcription elongation (Fig. 7A and B); however, it reduced the time that the elongation complex spent at the attenuation site (Fig. 7C and A; compare lanes 5 to 8 with lanes 1 to 4). The addition of either TFIIF or TFIIX resulted in a complete elimination of pausing by the polymerase at the attenuation site (Fig. 7A and C) as well as a stimulation of the rate of transcription elongation (Fig. 7B). The rate of elongation in the presence of TFIIF or TFIIX was approximately sixfold greater than that seen in the absence of any added factor or in the presence of TFIIE (Fig. 7A, lanes 1 to 4 and 17 to 20; Fig. 7B and C). These results indicate that TFIIF and TFIIX are factors which affect both the rate and efficiency of elongation, whereas TFIIS only prevents pausing. This latter conclusion is in agreement with previous studies (37), which demonstrated that TFIIS affected the efficiency by which RNA polymerase II passed through pausing sites. The effect of TFIIX during elongation was not due to contaminating TFIIF, as the TFIIX preparation failed to functionally replace TFIIF in the specific transcription reaction and also failed to react, in a Western immunoblot analysis, with antibodies directed against RAP 30, the small subunit of TFIIF (12).

TFIIS cannot prevent the elongation block conferred by a synthetic attenuation site composed of eight uridine residues. We were interested in exploring the effects of these factors on a stronger transcription elongation block. For this purpose, we used a template that carries the Ad2 MLP followed by a synthetic attenuator of eight consecutive T residues on the sense DNA strand. The construction of this template is described elsewhere (3). Incubation of this DNA template with a crude extract and labeled nucleotides, as described in Materials and Methods, resulted in the formation of pulse-labeled transcription complexes that could be purified by chromatography on a Sephacryl S-1000 column performed in 0.3 M KCl. The purified transcription complexes could be chased to generate transcripts of 450 (runoff) and 168 (attenuated) nucleotides (Fig. 8A). We next used this DNA construct to analyze the effects of TFIIF, TFIIS, and TFIIX.

Surprisingly, TFIIS was not able to promote elongation of the attenuated RNA on this DNA template (Fig. 8B, lanes 2 to 4). This result was not due to the effect of factor concentration; the addition of a 10-fold excess over the concentration needed to overcome pausing at the natural attenuation site present in the MLP also failed (data not

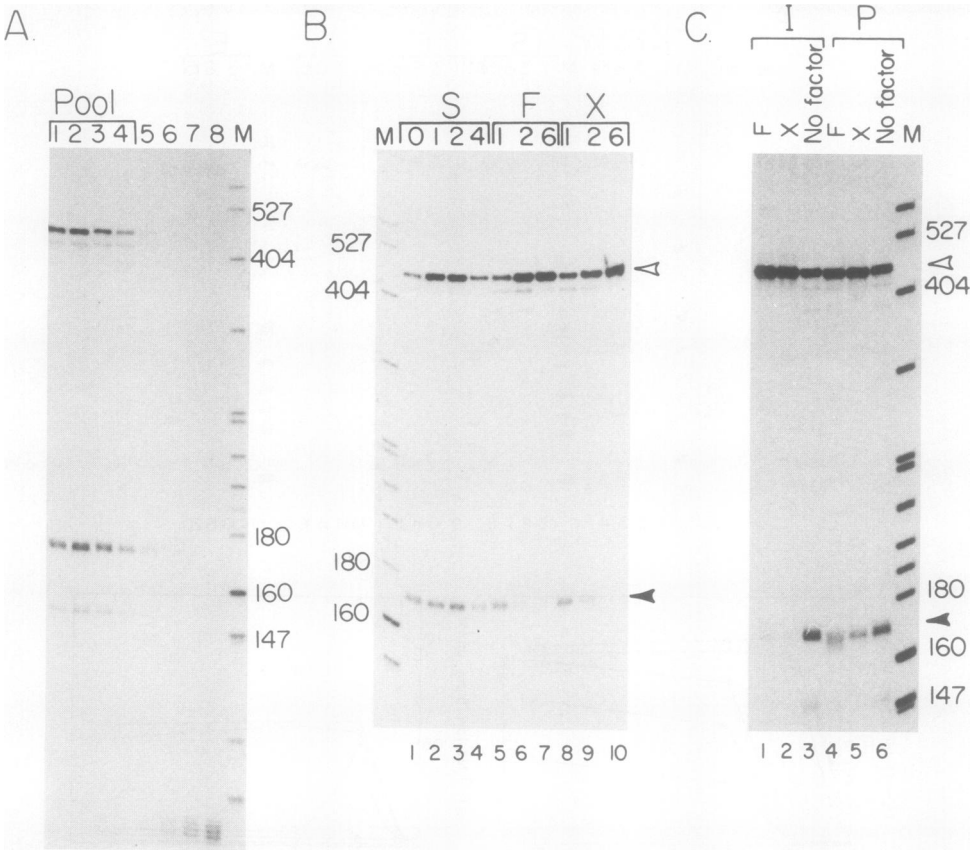

FIG. 8. Evidence that TFIIS cannot prevent the block of transcription elongation at the synthetic attenuation site. Transcription from the template bearing a synthetic attenuation site and purification of the briefly initiated complexes were as described in Materials and Methods. (A) Aliquots of the column fractions were incubated under chase conditions for 20 min, and the reaction products were analyzed by polyacrylamide gel electrophoresis. Fraction numbers are indicated at the top. In a parallel experiment, fractions 1 to 4 were pooled before the chase reaction and used as the source of elongation-competent complexes utilized in the experiments shown in panels B and C. (B) TFIIS, TFIIF, and TFIIX (S, F, and X) were added to transcription elongation mixtures at different concentrations (microliters) as indicated at the top, and elongation was allowed to proceed for 20 min. (C) Lanes labeled I represent reactions in which TFIIF and TFIIX were added to pulse-labeled transcription complexes with the elongation chase mixture and the reaction mixtures were incubated for 20 min. Lanes labeled P represent reactions in which TFIIF and TFIIX were added to transcription complexes after the 20-min elongation. After the factors were added, the reaction mixtures were incubated for an additional 20 min (40 min total elongation time). Transcription elongation chases for 20 and 40 min without the addition of elongation factors are represented in the lanes labeled No factor. Open arrowheads represent runoff RNA, and filled arrowheads represent attenuated RNA. Lanes M, DNA size markers (indicated in nucleotides alongside the gels).

shown). On the other hand, the addition of TFIIF or TFIIX to the purified complexes prior to reaching the attenuation site prevented attenuation (Fig. 8B, lanes 5 to 10). The protein concentration needed to overcome pausing at this synthetic site was similar to that required when the natural site was used (see Fig. 4).

Next we analyzed whether TFIIF and TFIIX could release the block to elongation if the transcription complexes had already reached the attenuation site. The experimental approach was the same as that described for Fig. 6; the cold nucleotide mixture was added to the transcription complexes 20 min before the factors, which were then further incubated for 20 min. The factors were added in limiting concentrations, i.e., the minimal concentrations that allowed readthrough of the attenuation site when added at the beginning of the chase (Fig. 4). With use of the synthetic eight-T template, both factors could at best partially release the block to elongation from the attenuation site (Fig. 8C, lanes 4 and 5). This finding is different from the results obtained with the natural Ad2 MLP attenuation site, which showed that TFIIX and, to a much lesser extent, TFIIF were able to release the elongation block.

TFIIS is loosely bound to the elongation complex. In contrast to TFIIF, which is assembled on the transcription complex at the preinitiation step (12, 13) and acts as a stimulatory factor during elongation, TFIIS has no effect during initiation of transcription and binds to the large subunit of RNA polymerase II, probably during elongation and presumably at specific attenuation sites (34, 37). It was therefore of interest to compare the affinities of these two transcription factors to the elongation complex. TFIIS or TFIIF were added to the column-purified complexes, and the nascent RNA was allowed to elongate under standard conditions or following a 10-fold dilution of the elongation chase reaction mixture, the rationale being that the factor which is only loosely bound to the elongation complex will dissociate from it as a result of dilution. Dilution of the reaction by itself had no effect on the block to elongation at the attenuation site (Fig. 9; compare lanes Con. 1 and 2). However, TFIIS, which was able to prevent the block to

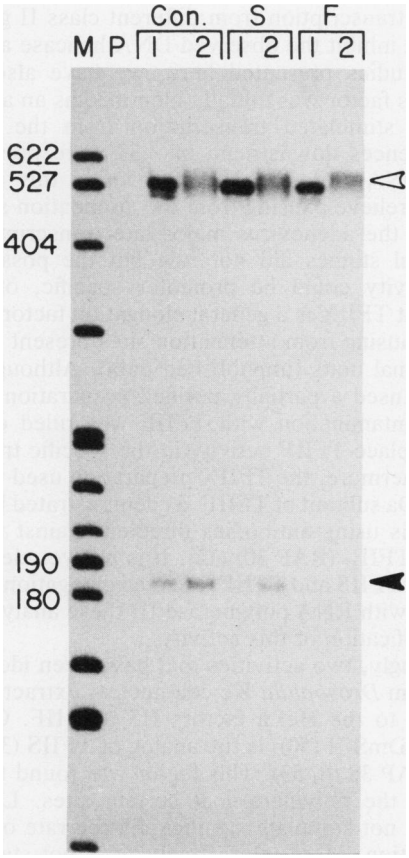

FIG. 9. Dissociation of TFIIS from the elongation complex upon dilution. Briefly initiated transcription complexes were purified by chromatography through a Sephacryl S-1000 column as described in Materials and Methods. The complexes eluted in the void volume were diluted to 360 μl with a buffer containing 20 mM Tris-HCl (pH 7.8), 6 mM $MgCl_2$, 10% glycerol, and 1 mM DTT to bring the final concentration of KCl to 50 mM. The minimal concentrations of TFIIS (S) and TFIIF (F) needed to release the elongation block were added to two reaction mixtures containing the column-purified complexes (in 50 mM KCl) and incubated at 30°C for 10 min. Then elongation chase mixture containing 500 μM ribonucleoside triphosphates was added to the same volume (lanes 1) or to a 10-fold-diluted reaction volume (lanes 2), and incubation was continued for additional 20 min. The labeled RNA was then extracted and analyzed by gel electrophoresis. Lanes: M, size markers (indicated in nucleotides at the left); P, pattern of the pulse-labeled RNA associated with the column-purified complexes; Con., pattern of the control RNA without the addition of factors; S and F, patterns of the RNA from the reaction mixtures to which TFIIS and TFIIF, respectively, were added.

elongation at the attenuation site, lost its activity when the reaction was diluted 10-fold (compare lanes S 1 and 2). On the other hand, TFIIF was active independently of whether the chase reaction was diluted (compare lanes 1 and 2 under F). As shown above, TFIIF is a stimulatory factor that may act during the entire elongation phase, while TFIIS is an antiattenuation factor that may act at specific sites only and then dissociate from the elongation complex.

DISCUSSION

Previous studies have used double-stranded DNA templates in which RNA polymerase II was forced to initiate transcription at a specific single-stranded poly(dC) tail (9, 20, 30, 31, 38, 50, 51). Those studies have shown that the poly(dC)-tailed template is an efficient artificial system with which to analyze transcription elongation and termination. By using that system, several intrinsic termination signals within human genes have been identified (22, 39), and factors that affect elongation by RNA polymerase II were characterized (13, 31, 38, 50). However, a limitation of this system is that the nascent transcripts often remain hybridized to the DNA template (9, 20). This phenomena is not observed in vivo and could interfere with regulatory events that occur during transcription elongation. Also, because 3′-end-extended poly(dC) templates initiate transcription in the absence of a promoter and auxiliary transcription factors, the effects of factors that modulate transcription elongation or termination in a promoter-dependent fashion have not been considered. It has recently been shown that at least one *cis*-acting promoter element present in the c-*myc* gene is required for transcriptional block (28). Therefore, studies on elongation and termination in a system in which initiation is promoter independent might bypass important regulatory processes.

In the studies presented here, we used an in vitro transcription system in which elongation-competent complexes were isolated and used to study the effects of different factors during elongation by RNA polymerase II. These elongation-competent complexes are ternary, consisting of a DNA template bearing a specific promoter, an RNA polymerase II molecule committed to elongation that is paused at a specific site as a result of nucleotide starvation, and a nascent RNA chain. The main features of this system are as follows. (i) It uses RNA polymerase II molecules that have accurately initiated transcription at a promoter. Therefore, the effect of accessory factors and *cis*-acting promoter elements on transcription elongation can be investigated. (ii) The elongation complexes can be efficiently separated from the majority of the proteins in one step by gel filtration chromatography in a buffer containing 0.3 M KCl. This salt concentration promotes the dissociation of factors known to interact with RNA polymerase II such as TFIIS (17, 37, 53), TFIIE (13, 36), and TFIIF (13). Thus, it allows the opportunity to study the effects on elongation of purified transcription factors. (iii) The purified transcription complexes displace the nascent RNA during elongation. (iv) The complexes pause at attenuation sites previously identified in vivo and not at any aberrant sites.

By using this system, two different activities that influence transcription elongation were observed: a readthrough activity that enables RNA polymerase II to overcome specific pause sites along the template, and a stimulatory activity that increases the rate of elongation. Our studies demonstrated that a highly purified preparation of TFIIS suppressed pausing by RNA polymerase II at specific sites and therefore decreased the length of time that the polymerase spent at the attenuation site present 185 nucleotides downstream of the Ad2 MLP cap site. TFIIS was capable of releasing pause at the attenuation site when added prior to or at the time the polymerase had reached the attenuation site. The same effect could be exerted by TFIIX and to a lesser extent by TFIIF. This observation suggests that these factors (TFIIF, TFIIS, and TFIIX) overcome pausing through different mechanisms. This notion is further supported by findings demonstrating that TFIIS does not enter into the transcription cycle via the preinitiation complex (34, 37) and therefore functions only during the elongation phase of transcription. However, TFIIF was found to be absolutely required for transcription and enters into the transcription

cycle via a direct interaction with RNA polymerase II prior to the initiation of transcription (12, 13). Moreover, TFIIS seems to function only when the polymerase has already paused at an attenuation site. TFIIS was found to specifically interact with purified RNA polymerase II (17, 37); therefore, we suggest that when the polymerase reaches the attenuation site, the polymerase exists in a pausing conformation. It is possible that TFIIS then induces a conformational change in the polymerase and/or the DNA template which allows the polymerase to resume elongation. The readthrough configuration of the polymerase is not retained during elongation; thus, TFIIS intervention with the polymerase is required at every attenuation site. This view is supported by the kinetic experiment in which the attenuated RNA was observed for a short period even though TFIIS was included in the chase reaction (Fig. 7) and by the finding that TFIIS is loosely bound to the elongation complex (Fig. 9). It should be expected that an activity capable of efficiently suppressing pausing should also stimulate the rate of elongation. However, our studies indicated that TFIIS failed to stimulate the rate of polymerization; this result could be due to the experimental conditions used, in which only one pausing site was available for release by TFIIS.

Our studies also demonstrated that a highly purified preparation of TFIIF (11) contained an activity that suppressed pausing and significantly stimulated the rate of elongation. The elongation effect by TFIIF is attributed specifically to TFIIF because addition of anti-RAP 30 antibodies inhibited the observed TFIIF-dependent elongation activity (Fig. 5). The antipausing activity observed in TFIIF (Fig. 4) is probably a consequence of the increased elongation rate that TFIIF confers to the polymerase (Fig. 7). This conclusion is based on the observation that if the polymerase reached the attenuation site in the absence of TFIIF, then the factor was no longer capable of effectively suppressing pausing. The fact that TFIIF could affect elongation after specific initiation had occurred, when RNA polymerase II was arrested during elongation as a result of nucleotide starvation, suggests that the entry of TFIIF into the transcription cycle does not necessarily have to occur via the preinitiation complex. However, our previous studies (12) as well as the studies of others (6, 7) have indicated that TFIIF is required for initiation of transcription and that it interacts with the preinitiation complex prior to initiation.

The mechanisms by which elongation factors increase the rate of polymerization are not known. It has been suggested that displacement of the RNA from the template might be in part responsible for this effect (51). It is of interest that TFIIF had a modest effect on elongation when transcription was performed under conditions in which the RNA remains hybridized to the DNA, using the poly(dC)-tailed template (13). Thus, it is possible that the configuration of the elongation complex influences the ability of TFIIF, and perhaps other factors, to productively interact with the elongating polymerase. Recent studies have suggested that an activity that bound to RAP 30 as well as to RNA polymerase II, probably RAP 74, contains a DNA helicase activity (52). However, the highly purified preparation of TFIIF used in our studies, which is composed only of two polypeptides with apparent molecular sizes of 30 kDa (RAP 30) and 78 kDa (probably RAP 74) (11), was devoid of ATPase and DNA helicase activities. Thus, it is unlikely that the RNA polymerase II-stimulatory activity associated with TFIIF is due to a DNA helicase activity. This view is in agreement with data presented by Sopta et al. (52), who found that antibodies against RAP 30, which coprecipitate RAP 74 (7) and inhibit transcription from different class II genes (6, 7, 12), did not inhibit the observed DNA helicase activity.

In the studies presented here, we have also analyzed TFIIX. This factor was initially identified as an activity that specifically stimulated transcription from the Ad2 MLP when sequences downstream of +33 were present in the template DNA (35). We have shown in this report that TFIIX can relieve pausing from the attenuation site present at +185 in the adenovirus major late transcriptional unit. While initial studies did not rule out the possibility that TFIIX activity could be promoter specific, our findings indicate that TFIIX is a general elongation factor capable of releasing pausing from attenuation sites present in different transcriptional units (unpublished data). Although in these studies we used a partially purified preparation of TFIIX, possible contamination with TFIIF was ruled out. TFIIX failed to replace TFIIF activity in the specific transcription assay; furthermore, the TFIIX preparation used was devoid of the 30-kDa subunit of TFIIF, as demonstrated by Western blot analysis using antibodies directed against the 30-kDa subunit of TFIIF (RAP 30) (12). It is not yet clear whether TFIIX, like TFIIS and TFIIF, affected elongation by directly interacting with RNA polymerase II; these analyses require further purification of this activity.

Interestingly, two activities that have been identified and purified from *Drosophila* Kc cell nuclear extracts appear to correspond to the HeLa factors IIS and IIF. One factor, designated DmS-II (50), is the analog of TFIIS (37), SII (34, 46), and RAP 38 (6, 53). This factor was found to suppress pausing of the polymerase at certain sites. Like TFIIS, DmS-II did not stimulate significantly the rate of transcription elongation. More interestingly, it is not stably associated with RNA polymerase II during elongation on 3′-end-extended poly(dC)-tailed templates (50). The second activity, factor 5, was found to stimulate the rate of transcription elongation. Furthermore, factor 5, like TFIIF, is required for specific transcription (31).

Whereas TFIIS acts only as an antitermination factor, both TFIIF and TFIIX markedly stimulate the rate of elongation. Both factors increased the rate of elongation to approximately 600 nucleotides per min. However, the in vivo elongation rate of the polymerase has been reported to be greater than 1,000 nucleotides per min (55). Therefore, it is possible that these and probably additional, yet unidentified factors control the rate of transcription elongation.

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