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The requirement for the basal transcription factor IIE is determined by the helical stability of promoter DNA

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The role of the basal transcription factor TFIIE was investigated in RNA polymerase II transcription reactions reconstituted with purified proteins. Using negatively supercoiled templates, which circumvent the requirement for TFIIH, we observed that transcription from the adenovirus major-late (ML) core promoter is more dependent on TFIIE than transcription from the adenovirus E4 (E4) or mouse mammary tumor virus (MMTV) promoters. For all three promoters, an increase in the ionic strength of the reaction mixtures led to an increased dependence on TFIIE. Analysis of hybrid ML/MMTV promoters showed that the region encompassing the start site, from −10 to +10, dictates this dependence. Transcription from a relaxed E4 template with a pre-melted −8 to +2 region was completely independent of both TFIIE and TFIIH. We propose that on negatively supercoiled templates TFIIE can facilitate promoter melting.

Key words: DNA topology/open complex/RNA polymerase II transcription/TFIIE/TFIIH

Introduction

Transcription initiation by RNA polymerase II is a multistep process that requires several basal transcription factors, which have been identified by *in vitro* reconstitution of transcription from the adenovirus major-late (ML) promoter (reviewed in Conaway and Conaway, 1993; Roeder, 1991; Zawel and Reinberg, 1992). The basal factor, nucleotide and DNA topology requirements of each step through which transcription proceeds have been partially elucidated. Assembly of the pre-initiation complex on DNA is an ordered process that begins with the binding of the TATA-binding protein (TBP) to its cognate element. For basal levels of transcription TBP alone suffices, whereas for high levels of activator-dependent transcription, the TFIID complex, which contains TBP and TBP-associated factors (TAFs), is required (reviewed by Tjian and Maniatis, 1994). The association of TAFs with TBP does not appear to influence the efficiency of the basal transcription reaction (Timmers and Sharp, 1991). Also, a comparison of TBP with TFIID complex in basal transcription shows a similar dependence on the other basal transcription factors (Parvin *et al.*, 1994). Subsequent to TBP, binding of TFIIB allows entry of TFIIF bringing with it RNA polymerase II (pol II) to form the DBpolF complex. TFIIE has been shown to join the DBpolF complex on its own and this event, in turn, allows TFIIH to enter the complex (Flores *et al.*, 1992). TFIIH has three ATP-dependent enzymatic activities: a CTD kinase and two separate DNA helicase activities (Feaver *et al.*, 1991; Lu *et al.*, 1992; Serizawa *et al.*, 1992; Ohkuma and Roeder, 1994; Schaeffer *et al.*, 1994). For the reconstituted *in vitro* assay, DNA helicase activity of TFIIH is the obligatory energy-requiring step, since inhibition of the CTD kinase activity does not affect the efficiency of the transcription reaction (Serizawa *et al.*, 1993). This DNA-helicase activity of TFIIH is thought to be involved in formation of the open complex (Schaeffer *et al.*, 1993). On promoter templates with a relaxed DNA topology, the transcription process requires TFIIE and TFIIH. In contrast, we have previously shown that negative supercoiling of the template alleviates the requirement for TFIIH activity and thus for hydrolysis of the β−γ phosphoanhydride bond of ATP (Timmers, 1994). Also, it has been demonstrated that the appearance of a potassium permanganate-hypersensitive region at the transcription start site of the adenovirus early region 4 (E4) promoter, indicative of single-stranded regions of DNA, is dependent on the presence of hydrolyzable ATP and transcriptional activators (Wang *et al.*, 1992a,b). Additionally, we have found that pre-melting the E4 promoter at the transcription start site circumvents the ATP energy requirement, possibly by obviating the need for TFIIH (Tantin and Carey, 1994).

It has become clear that different core promoters vary in their basal factor requirements. For example, Parvin and Sharp (1993) found that the supercoiled immunoglobin heavy chain (IgH) core promoter requires only TBP, TFIIB and pol II for transcription. An additional survey of 14 different core promoters indicated that the IgH promoter forms an exception as all the other promoters required TFIIF in addition to TBP, TFIIB and pol II for transcription (Parvin *et al.*, 1994). In the latter study, addition of TFIIE together with TFIIH led to increased levels of transcription in a promoter-dependent manner. In two other independent studies, TFIIE was also shown to be variably required for transcription from different supercoiled core promoters (Tyree *et al.*, 1993; Timmers, 1994). This is interesting because the latter observations were made in the absence of TFIIH. As the only substantiated role of TFIIE to date has been the recruitment of TFIIH (Flores *et al.*, 1992), these results indicate that TFIIE may also have an additional role in transcription that is core promoter dependent.

TFIIE is composed of two subunits, 34 and 56 kDa in mass, and exists as a heterotetramer in solution (Ohkuma *et al.*, 1990; Inostroza *et al.*, 1991; Peterson *et al.*, 1991).

Besides the recruitment of TFIIH (Flores *et al.*, 1992), TFIIE has also been shown to interact with pol II (Flores *et al.*, 1989). Recent binding studies have confirmed the interactions with pol II and TFIIH, and in fact show TFIIE to interact with all the basal transcription factors *in vitro*, resulting perhaps in a general stabilization of the transcription complex (Maxon *et al.*, 1994). Inspection of the amino acid sequence of the two TFIIE subunits has revealed intriguing regions of similarity with prokaryotic sigma factors (Ohkuma *et al.*, 1991; Peterson *et al.*, 1991; Sumimoto *et al.*, 1991). The 56 kDa subunit of TFIIE also possesses homology with the zinc ribbon region of the elongation factor TFIIS, which has been shown to bind single-stranded DNA (Qian *et al.*, 1993). The functional implications of these similarities have, however, not yet been determined.

In this study, we have further investigated the effect of TFIIE in transcription reactions in the absence of TFIIH. We found that stimulation of transcription by TFIIE was influenced by conditions that affect the helical stability of the DNA template. Additionally, we show that transcription from an E4 promoter template that contains a pre-melted start site is independent of TFIIE and TFIIH. Our data fit with a model in which under certain conditions TFIIE can facilitate promoter melting.

Results

TFIIE stimulation is promoter dependent

We have analyzed the effect of TFIIE and TFIIH on transcription from three different core promoters: the adenovirus ML, the adenovirus E4 and the mouse mammary tumor virus long terminal repeat promoter (MMTV). *In vitro* transcription reactions were reconstituted with recombinant bacterially derived TBP, TFIIB and TFIIE, baculovirus-expressed TFIIF, purified HeLa cell-derived TFIIH and either purified Chinese hamster ovary (CHO) cell pol II or immunoaffinity purified calf thymus pol II (see Materials and methods). Because we wished to analyze the effects of TFIIE, experiments were performed with limiting amounts of pol II and 2- to 3-fold saturating amounts of the basal factors as well as template DNA. Figure 1 shows the results of an *in vitro* transcription experiment which compares transcription from the three different promoters, which are linked to an identical guanosine-less (G-less) transcription cassette. For all three linearized promoters, TFIIH is absolutely required for transcription (Figure 1, lanes 5, 11 and 17), whereas with the supercoiled templates TFIIH can be omitted without significant loss of transcription (lanes 2, 8 and 14). Omission of TFIIE in the absence of TFIIH has little effect on the E4 and MMTV promoters (lanes 9 and 15), but severely reduces transcription from the ML promoter (lane 3). This result indicates that under these conditions TFIIE has an additional function besides allowing entry of TFIIH into the pre-initiation complex and that this function is promoter dependent.

TFIIE dependence increases with ionic strength

The variable effect of TFIIE on the three core promoters was subsequently assayed at different ionic strengths. Figure 2A shows that when the KCl concentration is increased, transcription becomes more dependent on the

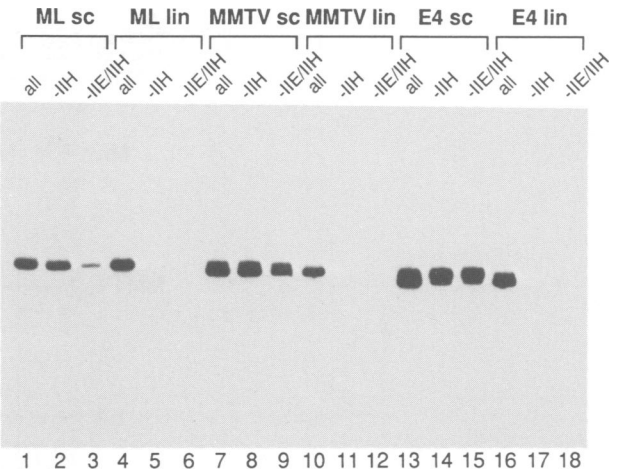

Fig. 1. Different core promoters exhibit different TFIIE requirements for basal transcription. For the experiment shown in this autoradiogram, transcription reactions were assembled with 100 ng of either pML(C_2AT)19Δ-51 containing the ML −53 to +10 region, pMV37 containing the MMTV LTR −37 to +10 region or pE4(C_2AT) containing the E4 −40 to +4 region, all driving a 380 nucleotide long G-less cassette, either supercoiled (sc) or linearized (lin) as indicated. In order to obtain comparable levels of transcription from the linear and supercoiled templates, in this experiment only, a 20 min pre-incubation of basal factors, pol II and template was included prior to the addition of nucleotides. Transcription was performed under the standard conditions as described in Materials and methods, i.e. 5 mM $MgCl_2$, 60 mM KCl. The reactions indicated with 'all' contained TBP, TFIIB, TFIIF, TFIIE, TFIIH and pol II. TFIIH was omitted from reactions analyzed in lanes 2, 5, 8, 11, 14 and 17. Both TFIIE and TFIIH were omitted from reactions of lanes 3, 6, 9, 12, 15 and 18, as indicated. Upon quantification by PhosphorImager analysis, no signal >0.1% of the maximal signal could be detected in lanes 5, 6, 11, 12, 17 and 18. The maximal level of transcription observed from the separate promoters differed <2-fold.

presence of TFIIE. Three independent experiments were quantified and the average levels of transcription are plotted in Figure 2B. The stimulatory effect of TFIIE on each promoter is depicted in Figure 2C. Between 20 and 60 mM KCl, both the MMTV and E4 promoters are relatively independent of TFIIE (1- to 2-fold stimulation). When the KCl concentration rises above 60 mM, both these promoters become increasingly dependent on TFIIE for efficient levels of transcription: 8-fold for the MMTV at 100 mM, 4- and 11-fold for the E4 at 100 and 120 mM KCl, respectively. The ML promoter is stimulated 4-fold by TFIIE at 20 mM KCl and becomes even more dependent at higher ionic strengths. In this assay, TFIIE has a 10-fold stimulatory effect on the ML promoter at 60 mM KCl. It is clear from these experiments that promoters that appear to be completely independent of TFIIE when assayed at low KCl concentrations become dependent on TFIIE for efficient transcription when ionic strength is increased. Furthermore, the ML, MMTV and E4 promoters can be classified as decreasingly TFIIE dependent. Interestingly, regardless of the presence of TFIIE, the optimal KCl concentration for transcription is successively higher for the ML, MMTV and E4 promoters (Figure 2B). The KCl concentration at which transcription can no longer be accurately detected is also correspondingly higher for the ML, MMTV and E4 promoters (Figure 2A and B).

The observations that TFIIE is increasingly required at higher ionic strengths and that the precise measure of

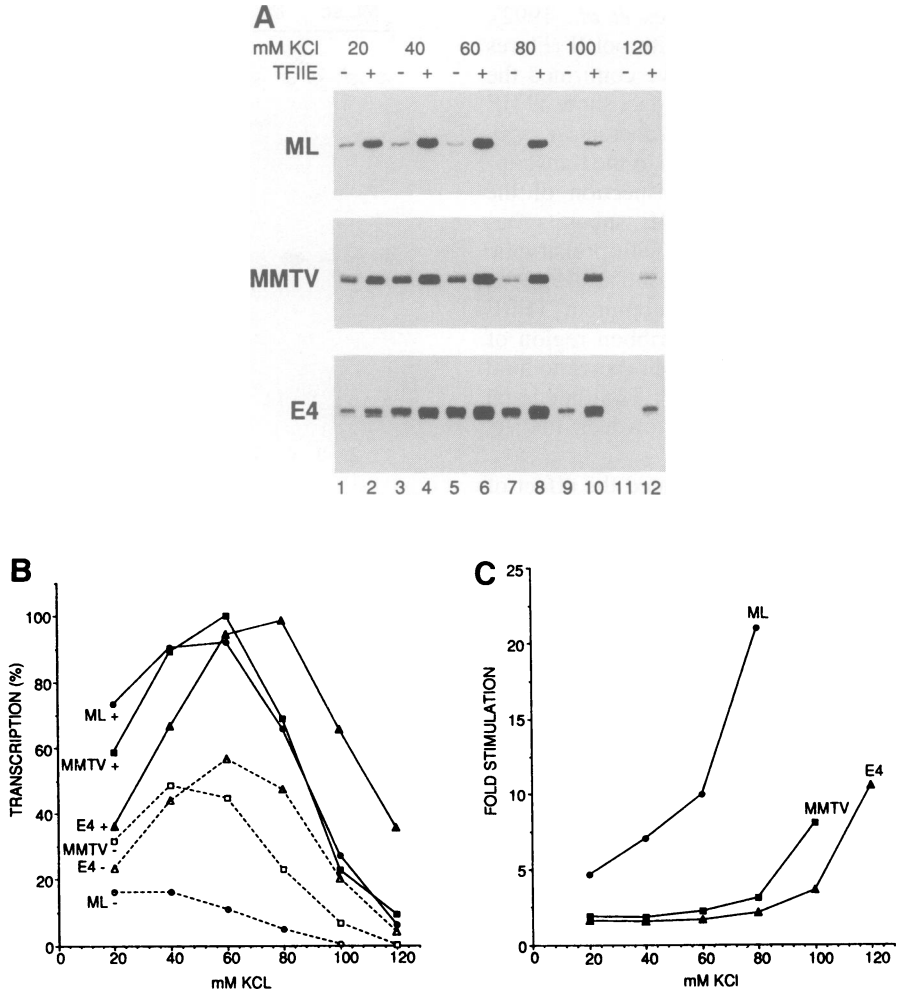

Fig. 2. At higher ionic strengths, transcription becomes increasingly dependent on TFIIE. (**A**) Transcription reactions using G-less cassette templates were assembled with increasingly higher concentrations of KCl as indicated. The $MgCl_2$ concentration was 5 mM. Reactions shown in the upper panel (ML) received 100 ng of supercoiled pML(C_2AT)19Δ-51, reactions of the middle panel (MMTV) were assembled with 100 ng supercoiled pMV37 and reactions of the lower panel (E4) received 100 ng supercoiled pE4(C_2AT). Transcription reactions of the even-numbered lanes (marked +) were assembled with TBP, TFIIB, TFIIF, TFIIE and pol II. Reactions shown in the odd-numbered lanes took place in the absence of TFIIE (−). (**B**) Three independent experiments were performed for each core promoter and quantified. The highest level of transcription in each experiment for a promoter was arbitrarily set at 100% and for each KCl concentration the average relative level of transcription is plotted. Transcription levels with the ML, MMTV and E4 promoters are indicated by circles, squares and triangles, respectively. Open symbols with dashed lines show relative transcription levels without TFIIE (−), black symbols with unbroken lines show relative levels of transcription in the presence of TFIIE (+). (**C**) The level of transcription in the presence of TFIIE was divided by the level of transcription in the absence of TFIIE to obtain the fold stimulation at each different KCl concentration. The average results from three determinations for each point are plotted. Because transcription levels in the absence of TFIIE fall to near background values, it was not possible to accurately determine the fold stimulation at KCl concentrations >80, 100 and 120 mM for the ML, MMTV and E4 promoters, respectively. SDs of the average values plotted in (B) and (C) were no higher than 31% of the averages, except for the ML promoter at 80 mM KCl (SD 45%) and for the E4 promoter at 120 mM KCl (SD 51%).

stimulation is promoter dependent indicated that DNA helical stability may influence the stimulation by TFIIE. It is unlikely that the salt-dependent effect of TFIIE is a stabilization of protein−protein contacts, which have become limiting for pre-initiation complex formation under conditions of higher salt, because all basal factors are supplied in excess. Another variable condition that would be expected to affect the helical stability of promoter DNA is the $MgCl_2$ concentration (Record, 1975). For the ML promoter, the effect of varying the $MgCl_2$ concentration in the absence or presence of TFIIE is shown in Figure 3A−C. Elevated $MgCl_2$ concentrations indeed increase the dependence on TFIIE for efficient transcription. The same is true for the MMTV and E4 promoters, whereby the promoters showed the same order of decreased TFIIE dependence for transcription as in the experiments performed with various KCl concentrations (data not shown). We note that a small increase in ionic strength can have a large effect on transcription efficiency (Figures 2B and 3B). This is interesting because small increases in salt concentration have been shown to strongly suppress helix opening of supercoiled DNA (Bowater *et al.*, 1994).

Besides negative supercoiling and ionic strength, temperature is a third variable which influences DNA helical stability (Geiduschek, 1962). The transcription experiments shown here were performed at the standard temperature of 30°C. All three promoters showed an increased TFIIE dependence at the lower temperature of 23°C (data

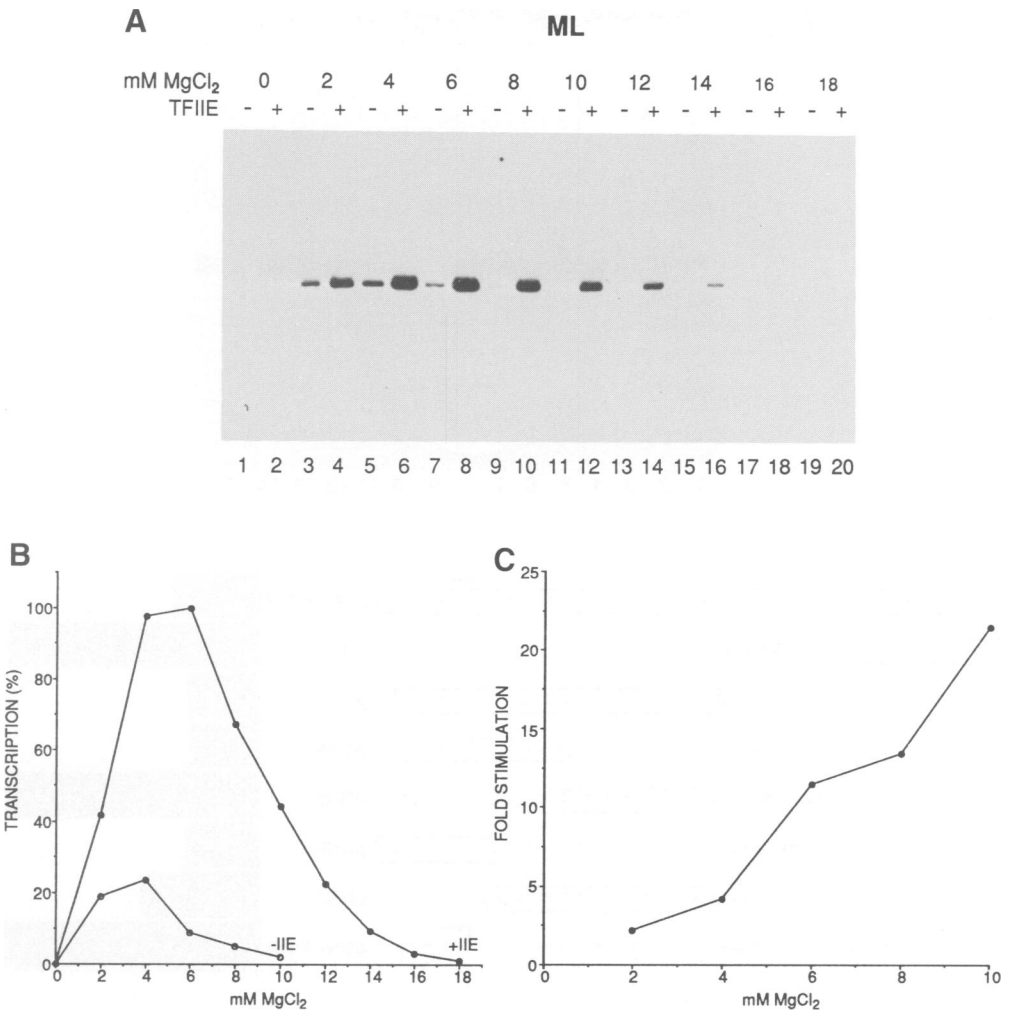

Fig. 3. TFIIE dependence increases with higher $MgCl_2$ concentrations. (**A**) Transcription reactions using the supercoiled pML(C_2AT)19Δ-51 plasmid containing a G-less cassette were assembled with increasing $MgCl_2$ concentrations as indicated. The KCl concentration was 60 mM. Similar to Figure 2, reactions marked '–' received no TFIIE, whereas reactions marked '+' did receive TFIIE. (**B**) The experiment of (A) was quantified and the level of transcription at 6 mM $MgCl_2$ in the presence of TFIIE was set at 100%. Open circles represent the levels reached in the absence of TFIIE, closed circles show levels in the presence of TFIIE. (**C**) The TFIIE stimulation between 2 and 10 mM $MgCl_2$ was obtained by dividing the level of transcription in the presence of TFIIE (+) by the level in the absence of TFIIE (−). TFIIE stimulation at $MgCl_2$ concentrations >10 mM could not be accurately determined because transcription levels in the absence of TFIIE fell to background values.

not shown), in agreement with the hypothesis that TFIIE stimulation is influenced by the helical stability of promoter DNA.

TFIIE dependence is determined by the region around the start site of transcription

Under standard conditions (60 mM KCl, 5 mM $MgCl_2$, 30°C), there is a differential requirement for TFIIE between the ML and MMTV promoters, which allows mapping of the promoter region that is responsible for TFIIE stimulation. ML/MMTV hybrid promoters, kindly made available by J. and R.Conaway (Conaway *et al.*, 1990), show that the −10 to +10 region dictates TFIIE stimulation (Figure 4). Primer extension products from transcripts of these promoters formed in the absence and presence of TFIIE are shown in Figure 4A. The sequence of the promoter constructs and the TFIIE stimulation are shown in Figure 4B. The MMTV and ML promoters again show a differential TFIIE dependence in this assay (lanes 1 and 2 versus lanes 15 and 16). The MMTV promoter exhibits nearly no stimulation, while the ML promoter is 5-fold dependent on TFIIE. Note that the TFIIE dependence in this assay is lower for both promoters when compared with the experiments performed with the G-less cassettes (Figure 2). When the sequence upstream of the TATA box is exchanged between the two promoters, the TFIIE dependence is seen to be largely determined by the downstream region (pDN2 and pDN3). When smaller segments of the downstream region are swapped (pDN4 and pDN5), it is clear that the −10 to +10 region dictates the level of TFIIE dependence. The MMTV promoter's lack of TFIIE response can be more finely mapped to the −2 to +10 region (pDN6). The nearly reciprocal construct (pDN7) which has the ML −1 to +10 region shows an intermediate TFIIE dependence. Taken together, these data indicate that the whole −10 to +10 region determines the level of TFIIE dependence.

This analysis shows that it is the sequence of the promoter region where open complex formation takes place that dictates the level of TFIIE dependence. This,

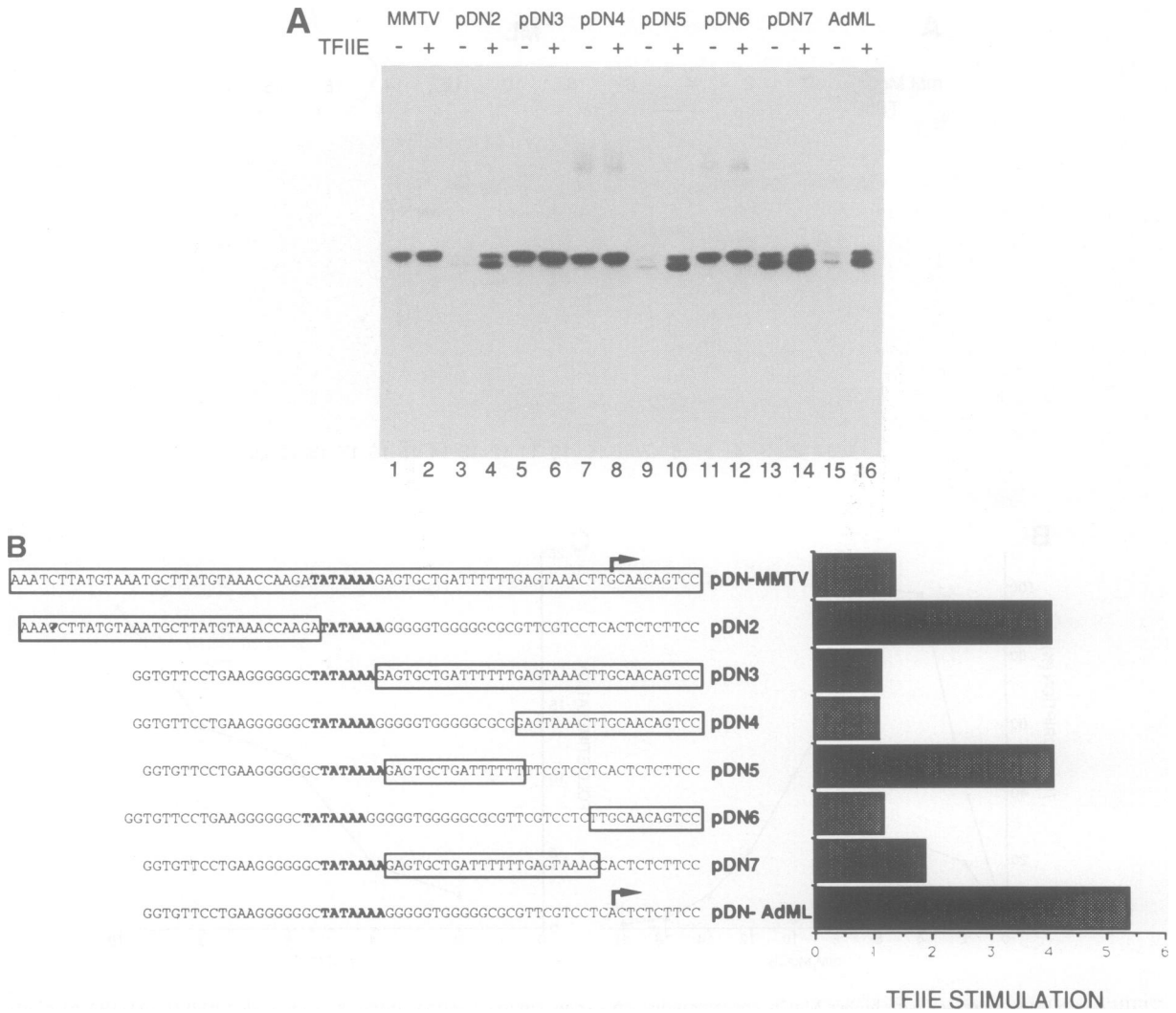

Fig. 4. The promoter region around the start site of transcription determines the level of TFIIE dependence. (**A**) Transcription reactions were assembled with different supercoiled ML/MMTV hybrid promoters as indicated, the sequence of which is shown in (B). Assay conditions were standard, i.e. 60 mM KCl, 5mM $MgCl_2$. Reactions of lanes marked (−) received no TFIIE. Reactions of lanes marked (+) did receive TFIIE. Transcripts were analyzed by primer extension with the M13 oligonucleotide as described in Materials and methods. Correct initiation and complete primer extension results in a 63 nucleotide product. The promoters with a ML start site yield a doublet reverse transcriptase product (pDN2, pDN5, pDN7 and pDN-AdML). Co-migrating sequence markers show the lower band to correspond to the +2 position (data not shown). It is not clear whether the +2 band represents a bona fide start site or whether it is due to incomplete extension by reverse transcriptase. Both bands were included in the quantification. (**B**) For each promoter construct, the TFIIE stimulation assay shown in (A) was performed three times. Experiments were quantified and the average TFIIE stimulation of each promoter construct is depicted on the right-hand side. SDs were on average 17% and did not exceed 29% of the average value for all promoters, with the exception of pDN-AdML (SD 38%). The left-hand side shows the sequence of each hybrid promoter aligned on their respective start sites (arrows). Regions derived from the MMTV promoter are boxed and TATA elements are shown in bold.

in combination with the data showing that the effect of TFIIE is increased by conditions that stabilize the DNA helix, strongly suggests a role for TFIIE in promoter melting.

A heteroduplex promoter is independent of TFIIH and TFIIE

TFIIE has a stimulatory effect in transcription from negatively supercoiled promoters in the absence of TFIIH. If the additional role of TFIIE is indeed to facilitate promoter melting, then the requirement for TFIIE should be completely obviated in transcription from a template with a pre-melted start site. We have shown that a linear E4 promoter with a heteroduplex −8 to +2 region (depicted in Figure 5A) circumvents the requirement for hydrolysis of the β−γ phosphoanhydride bond of ATP (Tantin and Carey, 1994), possibly by obviating TFIIH. By studying the basal factor requirements of the heteroduplex template, we sought to verify this prediction and, in addition, investigate how pre-melting of the template influences TFIIE stimulation.

Figure 5B shows that transcription from the pre-melted template is independent of both TFIIH and TFIIE. Lane 1 shows primer extension products from transcripts formed by transcription from the linear, wild-type E4 promoter template. Transcription starts from around two predominant sites: the first T of the T-stretch and from the A at position +1. This pattern is in agreement with earlier *in vivo* and *in vitro* studies on the E4 promoter start site (Baker and Ziff, 1981; Samuels *et al.*, 1984). As expected,

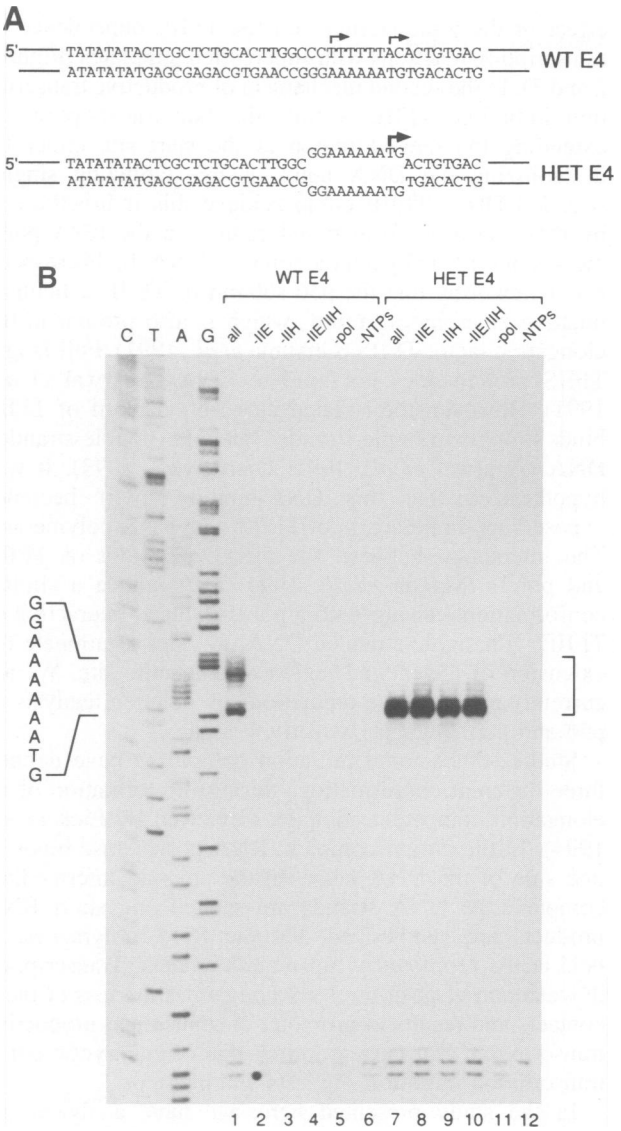

Fig. 5. Transcription from a promoter with a pre-melted start site does not require TFIIE or TFIIH. (**A**) The sequence of the wild-type (WT) E4 core promoter and of the heteroduplex template (HET) that contains a 10 bp mismatch from the −8 to +2 positions. These numbers are according to previous mapping of the E4 start site by RNA fingerprint analysis (Baker and Ziff, 1981). The heteroduplex template was generated as described previously (Tantin and Carey, 1994). Note that the template strand of the heteroduplex template has the same sequence as the wild-type promoter. Arrows depict the major start sites as observed in the primer extension experiments. (**B**) Transcription reactions were assembled with 100 ng linear E4 wild-type (WT) and heteroduplex (HET) promoters under standard conditions (60 mM KCl, 5 mM $MgCl_2$). The reactions of lanes 1 and 7 received the standard mix of transcription factors including TFIIH (all). TFIIE was omitted in lanes 2 and 8, TFIIH was omitted in lanes 3 and 9 and both TFIIE and TFIIH were omitted in lanes 4 and 10, as indicated. Lanes 5 and 11 received no pol II, and lanes 6 and 12 received no nucleotides. Transcripts were analyzed by primer extension with the E4 oligonucleotide as described in Materials and methods. Extension to +1 results in the 92 nucleotide product seen migrating as the lower band in lane 1. The lanes marked C, T, A and G show sequence markers generated with the same primer as used in the primer extensions. The pre-melted part of the sequence of the bottom strand is depicted to the left. The bracket to the right shows the portion of each lane that was taken for quantification. If the transcription level of lane 1 is set at 100%, then lanes 7, 8, 9 and 10 showed 103, 149, 98 and 129% of this level, respectively. All other lanes showed <3% of the signal of lane 1.

when either TFIIE or TFIIH are omitted, transcription is completely abolished (lanes 2 and 3, for quantification see the legend). On the heteroduplex template, which has a 10 bp mismatch extending 2 bp past either end of the T-stretch, transcription is almost exclusively initiated from the downstream start site. This is unlikely to represent an artefact created by reverse transcriptase since the sequences of transcripts from wild-type and heteroduplex templates are identical. It seems, therefore, that the position of the heteroduplex bubble influences the site of transcription initiation. The levels of transcription from the wild-type and heteroduplex templates are equal (see quantification of lanes 1 and 7) and omission of either TFIIE, TFIIH or both factors simultaneously shows that pre-melting the start site completely circumvents the requirement for TFIIE and TFIIH (lanes 8–10, for quantification see the legend). In fact, omission of TFIIE leads to a slight increase in transcription from the heteroduplex template (lanes 8 and 10 versus lanes 7 and 9). This is the opposite of the weak stimulation of transcription by TFIIE from the supercoiled E4 template under these KCl and $MgCl_2$ conditions. Also, at a higher ionic strength (100 mM KCl, 7 mM $MgCl_2$), transcription from the heteroduplex template is not dependent on TFIIE or TFIIH (data not shown), an additional argument against the effect of TFIIE being simply a result of pre-initiation complex stabilization at higher salt concentrations. The other basal factors, TBP, TFIIB and TFIIF, are still required for efficient levels of transcription (data not shown). This is not surprising since these factors are either directly or indirectly involved in recruiting pol II to the promoter. Omission of nucleotides in lanes 6 and 12 shows that the signals are derived from RNA generated during *in vitro* transcription. In a separate experiment, we found that the addition of 2 μg/ml α-amanitin completely abolished transcription from both templates (data not shown), which indicates that the RNA products are generated by pol II.

The results with the heteroduplex promoter show that a pre-melted start site obviates the need for those factors involved in promoter melting. These factors are TFIIH, required for its helicase activity, and TFIIE, required both indirectly for TFIIH recruitment and directly for a melting function, as made clear by its stimulation of transcription from supercoiled promoters in the absence of TFIIH.

Discussion

In an effort to understand the role of the basal transcription factor TFIIE, we have analyzed its effect on *in vitro* transcription reactions in the absence of TFIIH under a variety of experimental conditions. The presence of negative supercoils in the DNA template alleviates the requirement for TFIIH (Parvin and Sharp, 1993; Tyree *et al.*, 1993; Timmers, 1994), thus making it possible to distinguish between the role TFIIE has in recruiting TFIIH to the pre-initiation complex (Flores *et al.*, 1992) and additional functions of TFIIE. In contrast to transcription from the ML core promoter, we find that transcription from the E4 and MMTV promoters is independent of TFIIE under standard conditions, similar to observations made with the immunoglobulin heavy chain (IgH) core promoter (Parvin *et al.*, 1992). However, transcriptional dependence on TFIIE increases with all three promoters when the ionic

strength of the reaction is increased (Figure 2) or when the reaction temperature is lowered (data not shown). Both conditions reduce transient DNA melting by increasing the helical stability of DNA (Geiduschek, 1962; Schildkraut and Lifson, 1965). The experiments with the hybrid ML/MMTV promoters show that the region around the transcription start site determines dependence on TFIIE (Figure 4). This is the region where the DNA strands need to be separated before transcription can initiate. Indeed, TFIIE is not required for transcription from a promoter melted at the −8 to +2 region (Figure 5). Our study indicates that independent of TFIIH, TFIIE assists in melting of promoter DNA under certain conditions.

Results presented here and in other studies (Parvin and Sharp, 1993; Goodrich and Tjian, 1994; Timmers, 1994) indicate that for productive transcription pol II can use two different mechanisms *in vitro*. The difference depends on the topology of the DNA template. With relaxed templates, transcription requires the ATP-dependent DNA helicase activity of TFIIH and not its CTD kinase activity (Serizawa *et al.*, 1993). In this mechanism, TFIIE allows the entrance of TFIIH into the DBpolF complex (Flores *et al.*, 1992), possibly by protein−protein interactions (Maxon *et al.*, 1994). Our results also support the idea discussed by Goodrich and Tjian (1994) that TFIIE recruits TFIIH by creating a region of single-stranded DNA in the promoter. The second mechanism requires a negatively supercoiled DNA template and is independent of TFIIH activity (Parvin and Sharp, 1993; Tyree *et al.*, 1993; Timmers, 1994). We show that in this case the effect of TFIIE depends on parameters that determine the helical stability of promoter DNA. Under conditions of low ionic strength, the free energy stored in the negative supercoils probably induces transient melting of the DNA strands (reviewed by Lilley, 1988) which is sufficient for productive transcription. The propensity of a given promoter to melt at the transcription start site is determined by the local DNA sequence, thus accounting for the promoter-specific nature of this mechanism. The probability of the negatively supercoiled DNA duplex melting during transcription initiation is likely to depend on the relative stabilities of the single- and double-stranded isoforms. The survey of 14 different core promoters by Parvin *et al.* (1994) indicated that promoter activity in the absence of TFIIE and TFIIH is not simply a result of the G-C content of the −30 to +1 region of a promoter. To calculate the stability of single-stranded regions, we have used a computer program developed by Kowalski and co-workers, in which stacking interactions between nearest-neighbor bases are also taken into account (Natale *et al.*, 1992). In this analysis, regions around the start sites of the E4 and MMTV promoters are indeed predicted to have a lower energy requirement for melting of a 6–8 bp window in the −10 to +10 region than the region around the ML start site. However, the TFIIE-independent IgH promoter is predicted to require a similar amount of energy to melt as the TFIIE-dependent ML promoter (data not shown). This suggests that additional interactions which are not included in these calculations may also determine the propensity of a promoter region to melt. Interestingly, it has been found that modest increases in salt concentration strongly suppress localized melting in supercoiled DNA (Bowater *et al.*, 1994) and this agrees well with the strong effect of the ionic strength on the TFIIE dependence of transcription reactions with supercoiled templates (Figures 2 and 3). In the second mechanism of productive transcription initiation, TFIIE would stimulate transcription by extending the melted region at the start site either by destabilizing the DNA helix or by stabilizing single-stranded DNA. TFIIE could achieve this indirectly, e.g. by inducing a conformational change in the RNA polymerase or directly by interaction with DNA. In this respect, it is noteworthy that the p56 subunit of TFIIE contains a putative zinc-binding motif, which is also present in the elongation factor TFIIS (Ohkuma *et al.*, 1991). Full-length TFIIS protein does not bind to DNA (Agarwal *et al.*, 1991). However, the isolated zinc ribbon motif of TFIIS binds strongly to single-stranded but not to double-stranded DNA (Agarwal *et al.*, 1991; Qian *et al.*, 1993). It was hypothesized that this DNA-binding motif becomes exposed upon interaction of TFIIS with RNA polymerase. The interaction between the 56 kDa subunit of TFIIE and pol II (Maxon *et al.*, 1994) may induce a similar conformational change and a pol II-induced interaction of TFIIE with single-stranded DNA provides a rationale for extension of the melted region at a specific site. We are currently testing these predictions by deletion analysis of p56 and permanganate sensitivity assays.

Studies of bacterial initiation complexes have defined three different conformations during the formation of an elongation competent complex (reviewed by Eick *et al.*, 1994). In the closed complex, RNA polymerase binds to one side of the DNA helix. In the stressed intermediate complex, the DNA strands are melted and short RNA products are synthesized, but the RNA polymerase is held at the promoter by upstream contacts. Transcription downstream of position +9 accompanies the loss of these contacts and results in promoter clearance and productive transcription. Our current knowledge of eukaryotic pol II transcription initiation suggests similar steps.

In the study presented here, we have analyzed the production of full-length RNA transcripts and did not discriminate between abortive and productive initiation events. A recent study of abortive initiation by Goodrich and Tjian (1994) showed that TFIIE and TFIIH are not required for the formation of the first phosphodiester bond of RNA with relaxed and supercoiled templates; on relaxed templates, the DBpolF complex was sufficient for abortive initiation, but promoter clearance required TFIIE, TFIIH and ATP hydrolysis. In analogy with bacterial transcription, on relaxed templates the eukaryotic DBpolF complex would represent the stressed intermediate complex as it can produce only abortive transcripts. The DNA template strand needs to be exposed for abortive initiation, implying that the transcription complex is in an open conformation. However, the observation that this complex is incapable of promoter clearance indicates that the eukaryotic transcription complex must undergo further changes for the transition to a productive elongation complex. Thus, as is the case for prokaryotic transcription, the transition from a closed to a fully open complex on relaxed templates would require at least three steps. On supercoiled templates, the DBpolF complex could undergo the transition from a stressed intermediate to a productive complex with a frequency which depends on the helical stability of the promoter region. Association of TFIIE to the DBpolF

complex would increase this transition frequency by stabilizing or extending the melted promoter region in the direction of transcription. Interestingly, reports from D.Reinberg (L.Zawel and D.Reinberg, personal communication) indicate that TFIIE is dissociated from the transcribing complex when RNA polymerase reaches the +10 position. In the bacterial system, this is the site of transition from a stressed intermediate to a productive elongation complex (Straney and Crothers, 1987). Together, these results suggest that the transition to a productive elongating complex is mainly determined by the size of the melted region of DNA. A detailed analysis of the conformations of the eukaryotic pol II complex upon transition from the closed to the productive elongation complex will test this prediction and will show whether this comparison with the bacterial transcription complex is valid. The definition of more discrete stages through which open complex formation proceeds in the pol II basal transcription reaction may uncover new pathways of transcriptional regulation.

Materials and methods

Materials

Nucleotides and deoxynucleotides were of the Ultrapure grade and were purchased from Pharmacia/LKB. Radiolabeled [α-^{32}P]CTP (760 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. RNAguard and avian myeloblastosis virus (AMV) reverse transcriptase were from Pharmacia/LKB. Restriction endonucleases, *Escherichia coli* tRNA, T4 DNA polymerase and dithiothreitol (DTT) were from Boehringer Mannheim. HEPES, bovine serum albumin (BSA) and protease inhibitors were purchased from Sigma. Ultrapure ammonium sulfate was obtained from BDH. Phosphocellulose P11 was obtained from Whatman; the hydroxyapatite HTP cartridge was from BioRad. All other chromatography media and pre-packed columns were from Pharmacia/LKB. All pH values are given for the 1 M stock at room temperature, except for K_2HPO_4/KH_2PO_4 buffers, the details of which are given below.

Purification of transcription factors

Recombinant histidine-tagged human TBP (hisTBP), recombinant human TFIIB and recombinant human 34 and 56 kDa TFIIE subunits were expressed in *E.coli* and purified as described previously (Timmers, 1994).

Recombinant RAP30/74 (TFIIF) proteins were expressed in SF9 insect cells by co-infection with recombinant baculoviruses encoding RAP74 and RAP30, kindly provided by J. and R.Conaway (Aso *et al.*, 1994). Preparation of extract from 5 g of co-infected cells was performed as described for HeLa cells (Manley *et al.*, 1983) up to and including the 10% $(NH_4)_2SO_4$ extraction step. Proteins were selectively precipitated by stepwise addition of solid $(NH_4)_2SO_4$ to 38 and 65% saturation. Precipitated proteins were collected by centrifugation. TFIIF-containing fractions were identified by immunoblotting using rabbit polyclonal antibodies against human RAP30 (kindly provided by J.Parvin) and against human RAP74 (Santa Cruz Biotechnology, Inc.) and by Coomassie staining of SDS–polyacrylamide gels. TFIIF proteins exclusively precipitated in the 38–65% step. This protein fraction (310 mg) was adjusted by dialysis to buffer A [20 mM HEPES–KOH (pH 7.9)/20% glycerol/1 mM EDTA/1 mM DTT/0.5 mM phenyl methyl sulfonyl fluoride (PMSF)] plus 0.1 M KCl (A/0.1 M KCl) and applied to a 26 ml phophocellulose column. Bound proteins were eluted stepwise in buffer A/0.35 M KCl and in buffer A/0.6 M KCl. The 0.6 M KCl phosphocellulose fraction (20 mg protein) contained TFIIF and was adjusted to buffer B [20 mM Tris–HCl (pH 8.3)/20% glycerol/1 mM EDTA/1 mM DTT/0.5 mM PMSF] plus 0.1 M KCl. This fraction was loaded on a MonoQ HR5/5 column and the column was developed with a linear gradient from 0.1 to 0.5 M KCl in buffer B. RAP30/74 proteins eluted between 0.3 and 0.36 M KCl, and were ~95% pure at this stage. This fraction (5.2 mg protein) was applied to a HiLoad 16/60 Superdex 200 PG column equilibrated in buffer A/0.3 M KCl. The RAP30 and RAP74 proteins co-eluted as expected in fractions corresponding to ~180 kDa. The TFIIF preparation (4 mg protein) was homogeneous at this point, as evidenced by Coomassie staining of gels.

TFIIH was purified from HeLa whole-cell extract (Manley *et al.*, 1983) and purification was monitored by immunoblot analysis using the M.Ab.3C9 monoclonal antibody directed against the 62 kDa subunit of TFIIH (p62) (Fischer *et al.*, 1992) and rabbit polyclonal antiserum specific for ERCC3 (p89) (Ma *et al.*, 1994), as well as by stimulation of the linear template transcription reaction. Both subunits strictly co-eluted with TFIIH activity throughout the purification. This began with the 0.35–0.6 M KCl phosphocellulose fraction prepared as described previously (Samuels *et al.*, 1982). This fraction (200 mg protein) was dialyzed against T buffer [50 mM Tris–HCl (pH 7.9)/20% glycerol/1 mM EDTA/1 mM DTT/0.5 mM PMSF] plus 40 mM KCl (T/40 mM KCl) until conductivity corresponded to 50 mM KCl. Proteins were precipitated by the addition of solid $(NH_4)_2SO_4$ to 40% saturation. The pellet was resuspended in buffer T and 3 M $(NH_4)_2SO_4$ was added until the conductivity corresponded to 1 M $(NH_4)_2SO_4$. This fraction (100 mg protein) was loaded onto an 18 ml phenyl–Sepharose (Pharmacia high-performance grade) column (100×16 mm) and the column was developed with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in T buffer. TFIIH activity eluted between 100 and 10 mM $(NH_4)_2SO_4$. Peak fractions were pooled and aprotinin and pepstatin were added to 1 μg/ml. This was dialyzed against buffer T/0.1 M KCl and this fraction (9 mg protein) was applied to a MonoQ HR5/5 column. Bound proteins were eluted with a linear gradient from 0.1 to 0.6 M KCl in T buffer, and TFIIH activity eluted between 140 and 240 mM KCl. At this stage and on subsequent steps, aprotinin and pepstatin to 1 μg/ml were added to the eluted fractions. Half of each TFIIH peak fraction was pooled and the volume was doubled to accommodate the addition to final concentrations of 10 μM $CaCl_2$, 1 mM DTT and 20 mM K_2HPO_4/KH_2PO_4 (100 mM stock was pH 6.4 at room temperature). This preparation (2 mg protein, final pH 7.0) was loaded onto a 5 ml hydroxyapatite column and was developed with 20–600 mM K_2HPO_4/KH_2PO_4 (1 M stock was pH 7.0 at room temperature) in 10% glycerol, 10 μM $CaCl_2$, 1 mM DTT and 0.5 mM PMSF. Besides protease inhibitors, BSA to 0.2 mg/ml was added to eluted fractions and TFIIH eluted at 400 mM K_2HPO_4/KH_2PO_4. After dialysis against buffer T/0.05 M KCl, TFIIH was concentrated by a second MonoQ HR5/5 chromatography step as above. Besides BSA, this preparation was estimated to contain 0.025 mg/ml protein and was diluted 2.5 times with buffer A/0.1 mg/ml BSA before use in transcription reactions.

Two sources of pol II were used. The amanitin-resistant polymerase from the Ama1 CHO cell line was estimated to be >90% pure as judged by silver staining of SDS–polyacrylamide gels (Carthew *et al.*, 1988). The other pol II preparation was obtained by 8WG16 monoclonal antibody affinity purification from calf thymus extracts as described previously (Thompson *et al.*, 1990) with minor modifications suggested by N.Thompson and L.Strasheim (University of Wisconsin).

Preparations of basal factors and pol II were checked for cross-contaminating activities and for activities that altered the topology of DNA templates by means of immunoblot analysis, leave-out experiments and electrophoresis of DNA on agarose gels. Final preparations of each factor were adjusted to 100 mM KCl by dialysis or by dilution with buffer A/0.1 mg/ml BSA.

DNA templates

Supercoiled templates were prepared as described previously (Timmers and Sharp, 1991). Linearization was achieved with *Eco*RI digestion and template DNA was recovered by phenol-chloroform extraction, chloroform extraction, ethanol precipitation and resuspension in 10 mM Tris–HCl, 1 mM EDTA (pH 8.0). For G-less transcription reactions with the MMTV and E4 core promoters, the plasmids pMV37 and pE4(C_2AT) were constructed. p(C_2AT) plasmid DNA (Sawadogo and Roeder, 1985) was digested with *Sac*I, 3′ protruding ends were removed with T4 DNA polymerase and the vector was further digested with *Eco*RI. For pMV37, two complementary oligonucleotides, encompassing the −37 to +10 MMTV LTR sequence (Fasel *et al.*, 1982) with adenosine and thymidine at positions +1 and +7, respectively, were inserted. For pE4(C_2AT), complementary oligonucleotides, encompassing −38 to +4 of adenovirus type 2 early region 4 (Baker and Ziff, 1981), were inserted into the same vector.

The ML/MMTV hybrid promoter constructs were kindly provided by J. and R.Conaway (Conaway *et al.*, 1990). Fragments containing the E4 wild-type and heteroduplex templates were isolated as described previously (Tantin and Carey, 1994).

In vitro transcription reactions

Transcription reactions were assembled on ice and, unless otherwise indicated, contained 12 mM HEPES–KOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 12% glycerol, 0.6 mM EDTA, 0.3 mM PMSF, 1.2 mM DTT, 30–60 μg/ml BSA, 10 U of RNAguard and 100 ng of DNA

template in a 20 µl reaction volume. The ionic strengths of protein preparations and of solutions used to assemble transcription reactions were tested with a conductivity meter. One hundred nanograms of DNA template were saturating for all the transcription reactions whether the templates were supercoiled plasmids, linear plasmids or short promoter-containing fragments. Reactions employing G-less cassettes contained 60 µM ATP, 60 µM UTP, 10 µM [α-^{32}P]CTP (50 Ci/mmol) and 60 µM 3′-OMe-GTP. *In vitro* transcription reactions that were analyzed by primer extension contained 60 µM ATP, 60 µM UTP, 60 µM CTP and 60 µM GTP. Two- to 3-fold saturating amounts of basal factors were determined by titration with limiting amounts of pol II using pML(C_2AT)19Δ-51 (Sawadogo and Roeder, 1985) as template. The standard transcription reaction contained 200 ng His-TBP, 60 ng TFIIB, 150 ng TFIIE, 40 ng TFIIF and 0.25 µl CHO-derived pol II or 0.4 µl affinity-purified calf thymus pol II in a 20 µl reaction volume. A 0.75 µl volume of partially purified TFIIH was included where indicated. Originally, 50 ng TFIIE were determined to be 2- to 3-fold saturating at 60 mM KCl. When TFIIE titrations were repeated at 100 mM KCl, more TFIIE was required to reach saturation. Three-fold this amount (i.e. 150 ng) was used in all experiments shown here. Reaction mixtures were incubated for 60 min at 30°C. G-less transcription reactions were stopped and processed as described previously (Timmers and Sharp, 1991). Transcripts analyzed by primer extension were processed as described below.

Primer extension reactions

Transcription reactions were stopped by the addition of 180 µl TENS [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl, 0.1% SDS] and 5 µg *E.coli* tRNA. RNA products were phenol–chloroform extracted, chloroform extracted, 75 fmol [γ-^{32}P]ATP-labeled primer were added and this was precipitated by addition of ammonium acetate and ethanol. Pellets were resupended in 12 µl 2 mM Tris–HCl (pH 7.8), 0.2 mM EDTA, 250 mM KCl, 20 U RNAguard. This was heated to 68°C for 3 min and annealed either at 44°C for 60 min (M13 primer) or at 50°C for 20 min (E4 primer). Tubes were transferred to 42°C and 26 µl 50 mM Tris–HCl (pH 8.7), 10 mM $MgCl_2$, 5 mM DTT, 0.15 mg/ml BSA, 1.5 mM of each deoxynucleotide, 50 µg/ml actinomycin D, 20 U RNAguard and 9 U AMV reverse transcriptase were added. After an incubation of 45 min at 42°C, reactions were stopped by the addition of 165 µl TENS and 5 µg *E.coli* tRNA. After phenol–chloroform and chloroform extraction, nucleic acids were precipitated with sodium acetate and ethanol. The primer extension products were analyzed on denaturing 7% polyacrylamide gels. The M13 primer used for primer extension of the ML/MMTV hybrid promoters was 5′-GTTGTAAAA-CGACGGCCAGT and yields a 63 nucleotide product. The E4 primer used for primer extension from the wild-type and heteroduplex E4 promoters was 5′-GCGGCAGCCTAACAGTCAGCCTTACCAGTA and yields a 92 nucleotide product when transcription starts from the E4 promoter +1 position (Figure 5A).

Quantification of radioactive RNA and DNA products

Radioactive products were quantifed using a PhosphorImager gel scanner (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.25 software. An area completely covering the largest signal in a single experiment was taken as the standard size counting window. Background counts for each separate lane were obtained by applying this window above each signal and these counts were subtracted to counter the influence of lane-specific backgrounds.

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Since acceptance of this paper, Pan and Greenblatt (1994) *J. Biol. Chem.*, **269**, 30101–30104, have demonstrated that TBP, TFIIB, TFIIF and pol II support efficient transcription initiation from a heteroduplex AdML promoter. This is in agreement with the basal factor requirements of the heteroduplex AdE4 promoter presented here.