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Transcription initiation by RNA polymerase II does not require hydrolysis of the $\beta-\gamma$ phosphoanhydride bond of ATP

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When transcription by RNA polymerase II from the major-late (ML) promoter was studied with purified basal transcription factors, it was observed that transcription from negatively-supercoiled ML templates did not require transcription factor IIH (TFIIH). Addition of the basal factor TFIIE was highly stimulatory, but not absolutely required for this reaction. In contrast, transcription from relaxed or linear ML templates required both TFIIE and TFIIH. Adenylyl-imidodiphosphate (AMP-PNP), an ATP analog with a non-hydrolyzable $\beta-\gamma$ phosphoanhydride bond, could support RNA synthesis from supercoiled templates, but not from linear templates. Since AMP-PNP cannot act as a cofactor for the DNA helicase activity of TFIIH, this finding independently supported the conclusion that TFIIH is not required for transcription of negatively-supercoiled templates. Taken together, these data indicate that the ATP-dependent step in transcription initiation by RNA polymerase II is caused by a requirement for the ATP-dependent helicase activity of the basal factor TFIIH. The experiments also show that transcription initiation by RNA polymerase II does not require hydrolysis of the $\beta-\gamma$ phosphoanhydride bond of ATP *per se*.

Key words: ATP-dependence/basal transcription factors/DNA helicase/*in vitro* transcription/RNA polymerase II

Introduction

Transcription initiation by RNA polymerase II (pol II) in eukaryotes is a complicated process requiring multiple additional protein factors (for a review see Mitchell and Tjian, 1989). The transcription initiation complex is formed over the transcription start site by RNA pol II and the basal transcription factors. Several basal factors have been identified as essential components in reconstituted transcription assays employing DNA templates driven by the core promoter of the adenovirus major-late (ML) transcription unit (for reviews see Sawadogo and Sentenac, 1990; Zawel and Reinberg, 1992). Subsequent studies showed that the assembly of the initiation complex is an ordered process (for reviews see Roeder, 1991; Buratowski and Sharp, 1992), which starts with binding of the transcription factor IID (TFIID) to the TATA element of the ML core promoter. Binding of TFIIB to the TFIID–promoter complex allows the entry of TFIIF together with pol II. TFIIF seems to serve two functions: (i) it inhibits binding of pol II to non-promoter sites (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992) and (ii) it binds in association with the non-phosphorylated form of pol II to the TFIID–TFIIB–promoter complex (Lu *et al.*, 1991; Chesnut *et al.*, 1992). Subsequently, TFIIE binds to the preinitiation complex allowing entry of TFIIH (Lu *et al.*, 1991). One of the activities of the TFIIH factor (also known as BTF2 or δ from rat) is phosphorylation of the C-terminal domain (CTD) of pol II, converting it to the phosphorylated form (Lu *et al.*, 1992; Serizawa *et al.*, 1992). Evidence has been presented that this form of pol II is associated with elongation (Laybourn and Dahmus, 1990; Chesnut *et al.*, 1992). However, recent experiments have shown that inhibition of pol II phosphorylation has no effect on the transcription initiation or elongation efficiency of the ML promoter, indicating that pol II phosphorylation is not an obligatory step in the transcription reaction (Serizawa *et al.*, 1993). Nevertheless, it remains possible that under certain conditions and for a certain promoter phosphorylation of pol II by TFIIH is an essential step. Recently it was reported that a DNA helicase activity is a second interesting property of TFIIH. This activity could be responsible for the transition of the preinitiation complex to the open complex (Schaeffer *et al.*, 1993).

Previous experiments, both in crude extracts (Bunick *et al.*, 1982; Rappaport and Weinmann, 1987) and with partially purified basal factors (Sawadogo and Roeder, 1984; Conaway and Conaway, 1988), indicated that unlike other eukaryotic RNA polymerases (Bunick *et al.*, 1982; Lofquist *et al.*, 1993), transcription initiation by pol II requires hydrolysis of the $\beta-\gamma$ phosphoanhydride bond of ATP (reviewed in Conaway and Conaway, 1991). It was suggested that the observed energy-dependence could be due to the requirement for TFIIH — for its ATP-dependent DNA helicase activity and/or for the CTD kinase activity (Buratowski, 1993).

In this respect it is worth noting that transcription from the core promoter of the Ig heavy chain (IgH) gene can be reconstituted using only TATA-binding protein (TBP; the DNA-binding subunit of TFIID), TFIIB and pol II. The other basal factors (TFIIF, TFIIE and TFIIH) are not required on supercoiled IgH templates (Parvin *et al.*, 1992; Parvin and Sharp, 1993), suggesting that, contrary to ML promoter templates, formation of the open complex on IgH templates does not require the DNA helicase activity of TFIIH. Interestingly, progressive removal of negative supercoils from the IgH template rendered the transcription reaction increasingly dependent on TFIIE, TFIIF and TFIIH (Parvin and Sharp, 1993).

In the study described here, basal factors were prepared for reconstituted transcription assays. It was surprising to note that in the case of the ML promoter, preparations of TFIIH were not required for transcription of supercoiled ML promoter templates. However, when negative supercoils were removed, TFIIH was essential for transcription. This observation, that TFIIH is not required for transcription of

supercoiled templates, was supported by the finding that analogs of ATP containing a non-hydrolyzable $\beta-\gamma$ phosphoanhydride bond are efficient substrates for this transcription reaction.

Results

TFIIH is not required for reconstituted transcription of supercoiled templates

The basal transcription factors TFIIB, TFIIE and TBP used in this study were expressed in bacteria and purified to homogeneity (TFIIE and TFIIB) or to a high degree of purity (TBP). The pol II was purified from Chinese hamster ovary cells (Cartew *et al.*, 1988). When TFIIF was purified from whole-cell extracts of HeLa cells, it was surprising to find that ML promoter-directed transcription, using the negatively-supercoiled pML(C_2AT)19δ-51 as a template, could be reconstituted efficiently with the basal factors TBP, TFIIB, TFIIE, pol II and a highly-purified preparation of TFIIF. Transcription did not require a separate TFIIH-containing fraction. An obvious explanation would be that the other factors were contaminated with sufficient TFIIH activity to support the transcription reaction. However, no TFIIH could be detected in any of the basal factors or pol II preparations. This was based on immunoblotting experiments using the M.Ab.3C9 mAb (Fischer *et al.*, 1992) directed against the 62 kDa subunit of TFIIH (a kind gift from Drs R.Roy and J.-M.Egly) in which eight times the amount of a basal factor preparation used in the standard transcription reaction was analyzed (data not shown).

Recently it was found that TFIIH contains a DNA helicase activity (Schaeffer *et al.*, 1993) which could be responsible for melting DNA at the initiation site and, thus, the formation of the open complex. It may be possible that the free energy stored in the negative supercoils of the ML template could alleviate the requirement of TFIIH for transcription of these templates. This hypothesis was tested in reconstituted transcription of either supercoiled or linear guanosine-less templates yielding 380 nt transcripts using preparations of the various basal factors. The amounts of basal factors in the standard reaction were such that TFIIF activity was limiting and that the other factors, including the DNA template, were in 2- to 4-fold excess. Figure 1 shows that the omission of TFIIH reduces transcription of the supercoiled template only 2- to 4-fold (lanes 2 and 7), whereas without TFIIH no transcription of the linear template can be detected (lanes 10 and 15). Omission of TFIIE reduced transcription from the supercoiled template 20-fold and no transcription was observed from the linear template (lanes 5 and 13). Immunoblot analysis using a polyclonal antiserum specific for the 56 kDa subunit (kindly provided by Dr J.Parvin) showed no contamination of TFIIE in the other factor preparations (data not shown). When TBP, TFIIB, TFIIF or pol II were singly omitted, transcription from either template was eliminated (Figure 1, lanes 3, 4, 6, 8, 12–14 and 16). Addition of the relatively crude TFIIA/J preparation produced a weak inhibition of transcription (lanes 1 and 9). The supercoiled pMLC_2ATδ-71 plasmid yielding a 271 nt RNA product was included in the reactions as an internal control. Transcription reactions directed by this control template behaved similarly to the supercoiled pML(C_2AT)19δ-51 template except that in reactions also containing the linear pML(C_2AT)19δ-51

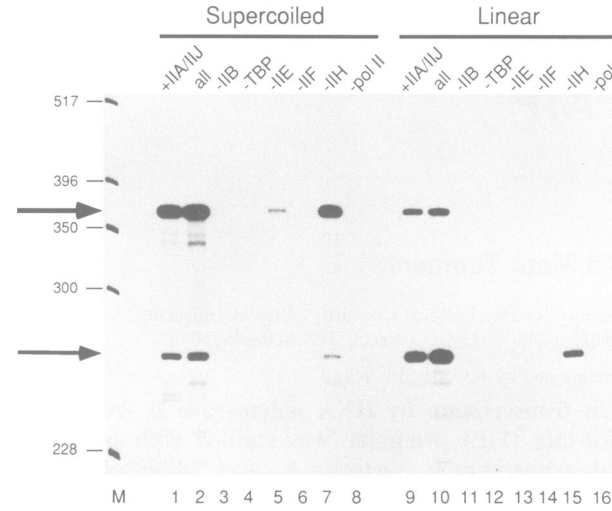

Fig. 1. The basal transcription factor TFIIH is not required for transcription of supercoiled templates. Transcription reactions were assembled using 100 ng of the plasmid pML(C_2AT)19δ-51, either supercoiled (lanes 1–8) or linear (lanes 9–16), yielding a 380 nt RNA product. As an internal control 50 ng of the supercoiled plasmid pMLC_2ATδ-71 was used yielding a 271 nt RNA transcript. The complete reaction (lanes 2 and 10) contained the basal transcription factors as described for the standard transcription reaction in Materials and methods. In addition to the standard set of factors, the reactions shown in lanes 1 and 9 received 0.5 μl (AB) fraction containing the transcription factors TFIIA and TFIIJ. In each of the other reactions one protein fraction was omitted as indicated above the lanes. The missing basal factors were as follows: TBP (lanes 3 and 11), TFIIB (lanes 4 and 12), TFIIE (lanes 5 and 13), TFIIF (lanes 6 and 14), TFIIH (lanes 7 and 15) or RNA pol II (lanes 8 and 16). The arrows indicate the position of correctly initiated transcripts. Lane M contained pEP40 DNA digested with *Hin*fI and radiolabeled by Klenow fragment using [α-^{35}S]dATP. The lengths of the DNA fragments are indicated to the left. The quantitation of radioactive RNA products by PhosphoImager analysis showed that the omission of either TFIIB, TBP, TFIIF or pol II resulted in a >100-fold reduction of transcription using either supercoiled or linear DNA templates. Addition of the TFIIA/TFIIJ resulted in a 30% reduction in transcription. In the transcription reaction with the supercoiled template, omission of TFIIH or TFIIE reduces transcription 2- to 4-fold or 20- to 40-fold, respectively. Transcription using linear templates without TFIIE or TFIIH is reduced 150- or 200-fold, respectively. The supercoiled pMLC_2ATδ-71, which served as an internal control, was preferred 2- to 4-fold over the linear template.

template the supercoiled control template was transcribed preferentially (Figure 1, compare lanes 1, 2 and 7 with lanes 9, 10 and 15). This agrees with the observation that the initiation complex is more rapidly formed on negatively supercoiled template when compared with relaxed templates (Mitzutani *et al.*, 1991).

The experimental results displayed in Figure 1 show that each of the basal factor preparations is free of cross-contamination by the other basal activities. Furthermore, the results indicate that transcription of negatively-supercoiled ML templates does not require TFIIH activity. Addition of TFIIH to this reaction resulted in a 2- to 4-fold stimulation. TFIIE has a strong stimulatory effect on these templates but is not absolutely required. In contrast, transcription of linear templates appears to require both TFIIH and TFIIE.

These experiments were elaborated by titration of TFIIH and TFIIE in transcription reactions using either linear or supercoiled ML template. Addition of TFIIH stimulated the reaction with supercoiled template only ~3-fold (Figure 2A, lanes 1–6). In contrast, no transcription of the linear

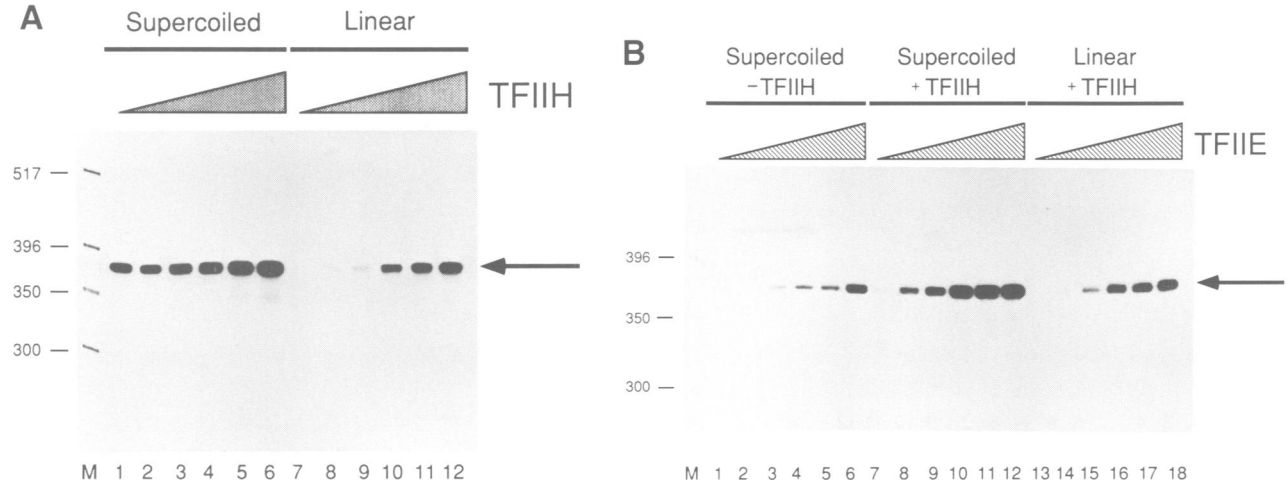

Fig. 2. Titration of the basal transcription factors TFIIH and TFIIE. The basal factors TFIIH (**A**) and TFIIE (**B**) were titrated in transcription reactions using supercoiled and linear pML(C_2AT)19δ-51 templates. (**A**) Transcription reactions lacking TFIIH were assembled using either supercoiled (lanes 1−6) or linear DNA template (lanes 7−12) and increasing amounts of the TFIIH fraction were included in the reaction. Control buffer was added in lanes 1 and 7. Reactions in lanes 2−6 received identical amounts of the TFIIH fraction as lanes 8−12: 0.22, 0.45, 0.90, 1.80 and 3.60 μl, respectively. The arrow indicates correctly initiated transcripts. Lane M contains comigrated DNA fragments of the indicated lengths. Quantitation of radioactive RNA products showed that transcription using the supercoiled template was stimulated 3-fold upon inclusion of TFIIH (comparing lanes 1 and 6), whereas the transcription reaction with the linear template was stimulated at least 50-fold (compare lanes 7 and 12). Maximum levels of transcription using supercoiled templates were 2-fold higher than with linear templates (compare lanes 6 and 12). (**B**) Transcription reactions lacking TFIIE were assembled using either supercoiled (lanes 1−12) or linear DNA template (lanes 13−18) and increasing amounts of bacterially-expressed TFIIE were added to the reaction. In lanes 7−18 standard amounts of TFIIH was also included. Reactions received control buffer (lanes 1, 7 and 13) or increasing amounts of TFIIE: 1 ng (lanes 2, 8 and 14), 3 ng (lanes 3, 9 and 15), 10 ng (lanes 4, 10 and 16), 30 ng (lanes 5, 11 and 17) or 100 ng (lanes 6, 12 and 18). The arrow indicates correctly initiated transcripts. Positions of comigrated DNA fragments are indicated to the left. The quantitation of radioactive RNA products showed that in the absence of TFIIH (lanes 1−6) transcription was half-maximal at 50 ng TFIIE, whereas in the presence of TFIIH transcription was half-maximal at 20 ng TFIIE (lanes 7−18). TFIIE stimulated transcription of supercoiled templates 10- to 20-fold (compare lane 1 with 6 and lane 7 with 12), whereas transcription from linear templates was stimulated at least 60-fold. Transcription was stimulated 3-fold by the addition of TFIIH (compare lane 6 with 12).

template was observed without TFIIH. Addition of this factor resulted in transcription levels similar to those of reactions using supercoiled templates (Figure 2A, compare lane 6 with 12). The basal factor TFIIE was titrated in transcription reactions using supercoiled or linear templates either in the presence or absence of TFIIH. Figure 2B again shows that in the absence of TFIIE, a low but detectable level of transcription can be observed in reactions with supercoiled templates (lanes 1 and 7). Transcription using these templates is stimulated 10- to 20-fold upon the addition of TFIIE (Figure 2B, lanes 1−12). Transcription with linear template is dependent on TFIIE (Figure 2B, lanes 13−18). Inclusion of the standard amount of TFIIH seemed to lower the amount of TFIIE required in the reaction. Half-maximal transcription was reached with 20 ng TFIIE, whereas 50 ng TFIIE was required in the absence of TFIIH. The observation that TFIIH reduces the amount of TFIIE required is in agreement with findings of Reinberg and co-workers (Flores *et al.*, 1992). The experiments shown in Figure 2A and B clearly demonstrate that transcription of the linear ML template requires the action of both TFIIE and TFIIH, whereas only TFIIE is required for transcription of negatively-supercoiled templates.

The requirement of basal factors for transcription of the ML promoter was investigated further by testing different topoisomers of the pML(C_2AT)19δ-51 plasmid. Relaxed template was prepared by incubation of supercoiled plasmid with wheat germ topoisomerase I. The template was isolated and the topology was analyzed by electrophoresis on agarose gels (data not shown). The resulting covalently-closed DNA was used in transcription reactions and compared with reactions using supercoiled or linear pML(C_2AT)19δ-51 templates. Figure 3 shows that as with the linear template, transcription of the relaxed template requires both TFIIE and TFIIH (lanes 1−8). This lends further support for the model that superhelicity of the ML template determines the requirement for TFIIH and TFIIE in the transcription reaction.

The ML promoter of adenovirus has served as the test promoter in the identification and purification of the basal transcription factors which has been accomplished in several laboratories. Recent transcription experiments by Parvin and Sharp using the core promoter of the IgH gene indicate that transcription using supercoiled IgH templates requires the action of only TPB, TFIIB and pol II. Removing negative supercoils rendered IgH transcription dependent on TFIIF, TFIIE and TFIIH (Parvin and Sharp, 1993). The finding that TFIIH is not required for transcription from ML template with negative supercoils raised the question whether this is a general phenomenon for all pol II-transcribed genes. Therefore, two additional supercoiled promoters were tested in the transcription reaction. Transcription of the mouse mammary tumor virus (MMTV) promoter dropped ~2-fold when TFIIH was omitted, and 6-fold when TFIIE was not present. Transcription was not reduced further when the reaction lacked both TFIIH and TFIIE (Figure 4, lanes 1−4). Omission of TFIIH led to an 8-fold reduction of transcription from the *Xenopus* albumin core promoter, and when TFIIE was omitted transcription was reduced 20-fold (Figure 4, lanes 5−8). As expected from previous studies (Parvin and Sharp, 1993), TFIIE and TFIIH had marginal effects on transcription from the IgH promoter (Figure 4,

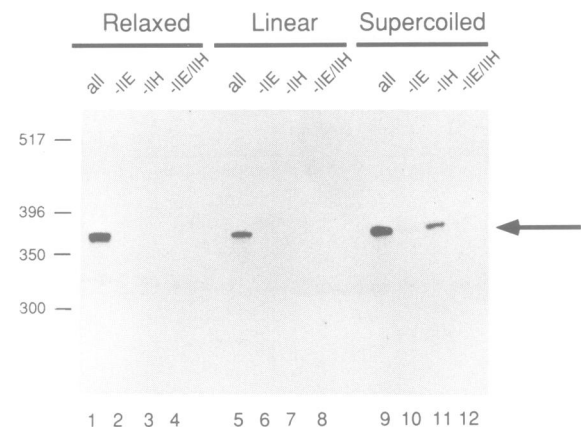

Fig. 3. The transcription of relaxed DNA templates has the same dependence on TFIIH and TFIIE as the transcription of linear DNA. Transcription reactions were assembled using relaxed (lanes 1–4), linear (lanes 5–8) or supercoiled pML(C_2AT)19δ-51 DNA template (lanes 9–12). The complete reaction (lanes 1, 5 and 9) contained the basal transcription factors as described for the standard transcription reaction in Materials and methods. In the other reactions, TFIIE (lanes 2, 6 and 10), TFIIH (lanes 3, 7 and 11) or TFIIE and TFIIH (lanes 4, 8 and 12) were omitted. The arrow indicates the position of correctly initiated transcripts. The positions of comigrated DNA fragments are indicated to the left. The quantitation of radioactive RNA products showed that transcription using supercoiled templates was 2.5-fold lower in the absence of TFIIH (lanes 9 and 11) and was ~12-fold lower in the absence of TFIIE (compare lane 9 with 10). The omission of TFIIH did not result in a further reduction of transcription (compare lane 10 with 12). Transcription using either relaxed or linear plasmid templates was reduced at least 20- to 60-fold when TFIIE or TFIIH are omitted from the reaction (compare lane 1 with 2–4 and lane 5 with 6–8).

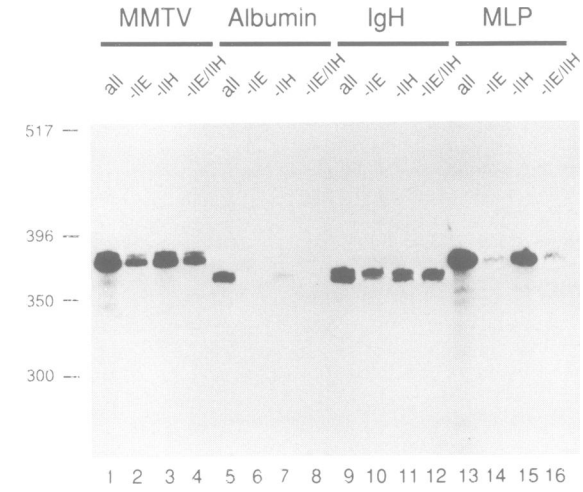

Fig. 4. TFIIE and TFIIH requirement of different core promoters. Transcription reactions were assembled using supercoiled DNA templates containing guanosine-less cassettes of varying length driven by different core promoters: the MMTV LTR (lanes 1–4), the *Xenopus* albumin gene (lanes 5–8), the IgH gene (lanes 9–12) and the ML transcription unit (lanes 13–16). The complete reaction (lanes 1, 5, 9 and 13) contained the basal transcription factors as described for the standard transcription reaction in Materials and methods, except that in lanes 1–12 the amount of TBP was increased 3-fold to compensate for the lower affinities of TBP for the TATA elements of these core promoters (unpublished observations). In the other reactions TFIIE (lanes 2, 6, 10 and 14), TFIIH (lanes 3, 7, 11 and 15) or TFIIE and TFIIH (lanes 4, 8, 12 and 16) were omitted. Positions of comigrated DNA fragments are indicated to the left. The quantitation of radioactive RNA products showed that the omission of TFIIE leads to a 6-, 25-, 2- or 30-fold reduction of transcription from the MMTV, albumin, IgH or ML promoters, respectively. The omission of TFIIH reduces transcription from the MMTV promoter 5-fold, from the albumin promoter 8-fold, from the IgH promoter 2-fold and from the ML promoter 3-fold. The omission of both TFIIE and TFIIH does not result in a further reduction in transcription than the omission of TFIIE alone.

lanes 9–12). The ML promoter was included in this experiment as a control (Figure 4, lanes 13–16) and showed the same pattern as observed in the previous experiments. The experimental results in Figure 4 show that different core promoters have different requirements for the basal factors TFIIH and TFIIE. Although the albumin promoter has a strong TFIIH dependence, transcription from all supercoiled templates in the absence of TFIIH activity could readily be detected. The differences in the dependence on TFIIH could be caused by differences in the tendency of these promoters to unwind at the transcription start site. We are currently determining which region of the core promoters is responsible for the requirement for TFIIE and TFIIH.

Hydrolysis of ATP is not essential for transcription initiation by RNA pol II

RNA synthesis requires hydrolysis of the $\alpha-\beta$ phosphoanhydride bond of the incoming nucleotide triphosphate. However, early studies have shown that transcription by RNA pol II also requires hydrolysis of the $\beta-\gamma$ phosphoanhydride bond of ATP. Analogs of ATP containing a non-hydrolyzable $\beta-\gamma$ bond were unable to support RNA pol II transcription (Bunick *et al.*, 1982). Subsequent experiments showed that this energy-dependent step occurred during the initiation reaction and that dATP could substitute for ATP (Sawadogo and Roeder, 1984; Rappaport and Weinmann, 1987; Conaway and Conaway, 1988). The two known activities of TFIIH, CTD kinase and DNA helicase activity, both require hydrolysis of ATP or dATP (Lu *et al.*, 1992; Serizawa *et al.*, 1992; Schaeffer *et al.*, 1993). Recently Conaway and co-workers used the kinase inhibitor H-8 to demonstrate that phosphorylation of the CTD of pol II is not required in a reconstituted transcription assay using linear templates (Serizawa *et al.*, 1993). These experiments were repeated and similar results showed that no effect of the H-8 kinase inhibitor could be observed on transcription of either supercoiled or linear templates in the presence of TFIIH (data not shown). These conclusions fit with the observations that TFIIH is not required for transcription of supercoiled templates (Figure 1).

This lack of TFIIH requirement raised the possibility that the transcription reaction can be supported by ATP analogs like adenylyl-imidodiphosphate (AMP-PNP) which do not contain a hydrolyzable $\beta-\gamma$ bond. This hypothesis was investigated and the results are shown in Figure 5. Transcription mixtures using guanosine-less transcription templates were assembled with UTP and CTP as the only nucleoside triphosphates. ATP and its analog AMP-PNP were added at varying concentrations. It was found that AMP-PNP efficiently supported transcription of the supercoiled ML template (Figure 5A, lanes 7–11). Maximum transcription levels with AMP-PNP or ATP were identical (Figure 5A, lanes 6 and 11). Addition of dATP to this reaction had no stimulatory effect, but resulted in a weak inhibition (Figure 5A, compare lanes 7–11 with lanes 12–16). No transcription could be detected without added ATP or with dATP alone (Figure 5A, lanes 1 and 17).

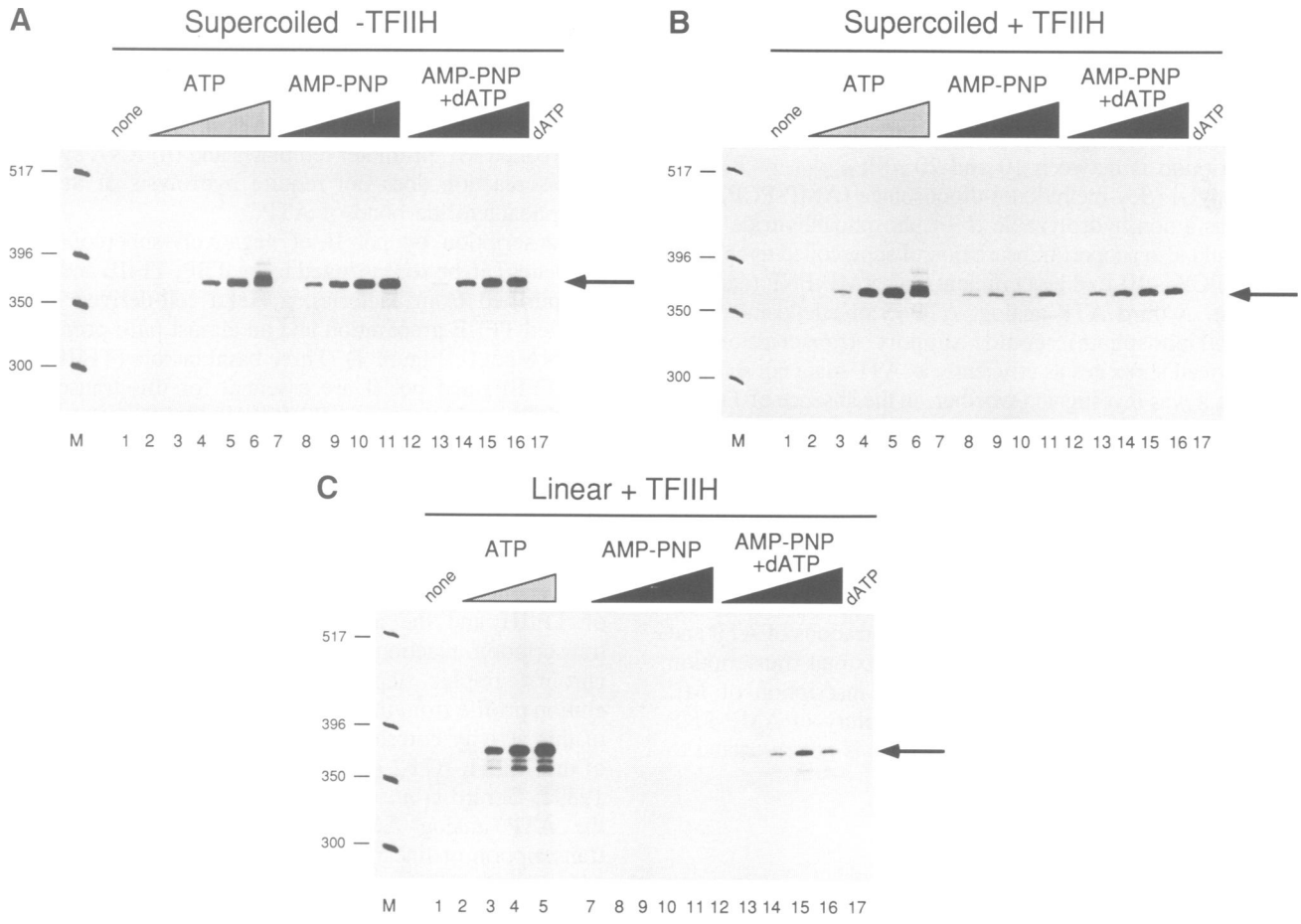

Fig. 5. Hydrolysis of the $\beta-\gamma$ phosphoanhydride bond of ATP is not required for transcription initiation. Transcription mixtures contained 10 μM CTP and 10 μM UTP instead of the standard nucleotides. **(A)** Nucleotide requirement of the transcription reaction using a supercoiled template in the absence of TFIIH. Reactions containing the basal transcription factors except TFIIH were assembled using supercoiled pML(C_2AT)19δ-51 template. The $MgCl_2$ concentration was raised to 7.5 mM to compensate for the high levels of nucleotides. Lane 1 received control buffer. In lanes 2–6 increasing concentrations of ATP were included: 1, 3, 10, 30 and 100 μM, respectively. The ATP analog AMP-PNP contains a non-hydrolyzable $\beta-\gamma$ phosphoanhydride bond and was included at 30 μM (lanes 7 and 12), 100 μM (lanes 8 and 13), 300 μM (lanes 9 and 14), 1 mM (lanes 9 and 15) and 3 mM (lanes 10 and 16). In addition, reactions in lanes 12–17 received 0.5 mM dATP. The arrow indicates the position of correctly initiated transcripts. Lane M contains DNA fragments of the indicated lengths. The quantitation of radioactive RNA products showed that transcription was half-maximal at 15 μM ATP or at 300 μM AMP-PNP. Transcription with dATP alone was $<0.1\%$ of the maximum level. **(B)** Nucleotide requirement of the transcription reaction from a supercoiled template in the presence of TFIIH. Reactions containing the complete set of basal factors were assembled as in A using supercoiled pML(C_2AT)19δ-51 template. The autoradiogram of A was exposed twice as long as B. The quantitation of radioactive RNA products indicated that transcription was half-maximal at 20 μM ATP, at 100 μM AMP-PNP or 200 μM AMP-PNP in the presence of dATP. Maximum levels of transcription with AMP-PNP in the absence of dATP are 3.5-fold lower and with AMP-PNP plus dATP are 2-fold lower when compared with transcription with ATP. **(C)** Nucleotide requirement of the transcription reaction from a linear template in the presence of TFIIH. Reactions containing the complete set of basal factors were assembled as in A using linear pML(C_2AT)19δ-51 template. The sample containing 100 μM ATP was lost during isolation of the radioactive products. The quantitation of radioactive RNA products indicated that transcription was half-maximal at 10 μM ATP or 300 μM AMP-PNP with dATP. Even after prolonged exposure no transcription can be detected with AMP-PNP alone (data not shown). Addition of dATP restores transcription to 30% of the level with ATP.

Transcription of the supercoiled template in the presence of TFIIH was also supported by AMP-PNP alone (Figure 5B, lanes 7–11). To compensate for the TFIIH stimulation of transcription from supercoiled ML templates (see Figure 2A), the autoradiogram of Figure 5B was exposed for 8 h instead of the 16 h of Figure 5A. Maximum transcription levels with AMP-PNP are ~3-fold lower than with ATP (Figure 5B, lanes 6 and 11). These data show that AMP-PNP can only support the TFIIH-independent transcription reaction and they fit nicely with the observation that addition of TFIIH resulted in a 3-fold stimulation of transcription from supercoiled templates (Figure 2A). Also compatible with this was the finding that the addition of dATP to transcription mixtures, including TFIIH, stimulated the reaction with AMP-PNP (Figure 5B, lanes 12–16). Maximum transcription levels with AMP-PNP and dATP were, however, lower than those with ATP alone. It is possible that while effective in supporting the helicase activity of TFIIH, the addition of 0.5 mM dATP results in competitive inhibition of the elongation by RNA pol II. In contrast to the transcription of supercoiled templates, reactions using linear templates were not supported by AMP-PNP alone (Figure 5C, lanes 7–11). However, the addition of dATP allowed transcription of the linear template (Figure 5C, lanes 12–16). This also renders it highly unlikely that transcription with AMP-PNP in the previous experiments is caused by an ATP contamination in the AMP-PNP preparations. [Identical results were obtained when AMP-PNP was purchased from another manufacturer (data not shown).] Under these conditions maximum transcription

levels with AMP-PNP and dATP are 3-fold lower than with ATP alone (Figure 5C, compare lane 5 with 15). The concentration of AMP-PNP for half-maximal transcription is 200−300 μM in reactions with either supercoiled or linear templates. The concentration of ATP for half-maximal transcription is between 10 and 20 μM.

Adenylyl (β,γ-methylene)-diphosphate (AMP-PCP) also contains a non-hydrolyzable β−γ phosphoanhydride bond and could also support transcription of supercoiled templates. AMP-PCP is 10-fold less efficient than AMP-PNP (data not shown). A third ATP analog, ATPγS or adenosine-5′-*O*-(3-thiotriphosphate) could support transcription of supercoiled templates as efficiently as ATP (data not shown).

Next it was investigated whether, in the absence of TFIIH, transcription of the MMTV and IgH core promoters of MMTV would be supported by AMP-PNP. Supercoiled DNA templates carrying the respective promoters were transcribed in the presence of varying concentrations of either ATP or AMP-PNP. Figure 6 shows that AMP-PNP can efficiently support transcription from both promoters. The maximum levels of transcription with AMP-PNP are identical to those with ATP. The concentrations of ATP and AMP-PNP required to achieve half-maximal transcription are similar to those observed with transcription of ML templates. These data show that the ability of AMP-PNP to support transcription by RNA pol II is not restricted to certain promoters.

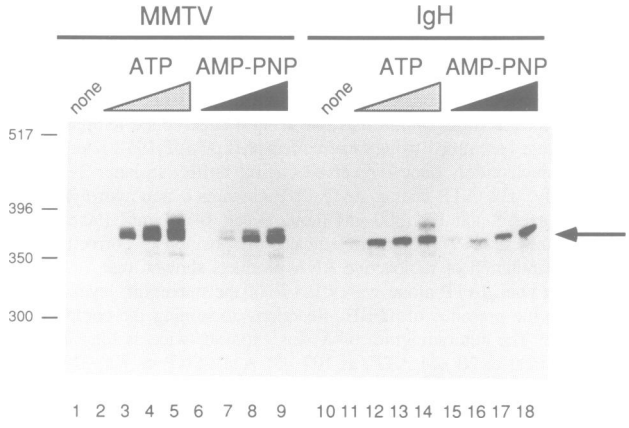

Fig. 6. Hydrolysis of the β−γ phosphoanhydride bond of ATP is not required for transcription from the MMTV and IgH core promoters. Transcription mixtures contained 10 μM CTP and 10 μM UTP instead of the standard nucleotides. Reactions containing the basal transcription factors except TFIIH were assembled using supercoiled templates either driven by the core MMTV promoter (lanes 1−9) or by the core IgH promoter (lanes 10−18). The amount of TBP was increased 3-fold compared with the standard reaction. Reactions in lanes 1 and 10 received control buffer. In the other lanes increasing concentrations of ATP or AMP-PNP were included. The concentration range for ATP was 1 μM (lanes 2 and 11), 3 μM (lanes 3 and 12), 10 μM (lanes 4 and 13) or 30 μM (lanes 5 and 14). The concentrations of AMP-PNP containing the non-hydrolyzable β−γ phosphoanhydride bond were 30 μM (lanes 6 and 15), 100 μM (lanes 7 and 16), 300 μM (lanes 8 and 17) and 1 mM (lanes 9 and 18). The arrow indicates the positions of correctly initiated transcripts. The quantitation of radioactive RNA products indicated that transcription of the MMTV promoter was half-maximal at 10 μM ATP or 200 μM AMP-PNP. Maximum levels of MMTV transcription with AMP-PNP are 75% of the maximum transcription levels with ATP. Transcription from the IgH promoter was half-maximal at 7 μM ATP or 100 μM AMP-PNP. Maximum levels of IgH transcription with AMP-PNP are identical when compared with transcription with ATP.

Discussion

The results presented in this study establish two points: (i) the basal transcription factor TFIIH is not essential for accurate transcription by RNA pol II of negatively-supercoiled ML promoter templates and (ii) RNA synthesis in this reaction does not require hydrolysis of the β−γ phosphoanhydride bond of ATP.

Transcription by pol II of negatively-supercoiled ML templates can be reconstituted using TBP, TFIIB and TFIIE (all purified from bacteria), a HeLa cell-derived highly-purified TFIIF preparation and an almost pure preparation of RNA pol II (Figure 1). Three basal factors (TFIIB, TBP and TFIIF) and pol II are essential for this transcription reaction. Addition of TFIIE is highly stimulatory. Transcription of ML templates which lack negative supercoils is not supported by this set of basal factors but requires the action of TFIIH (Figures 1 and 2). The identification of this activity as TFIIH (also known as BTF2 or δ from rat) is based on multiple observations. Firstly, a strict coelution between the presence of the 62 kDa subunit of TFIIH and the activity which stimulates the linear transcription reaction was observed in five consecutive chromatographic steps (data not shown). Secondly, the elution profile from the different chromatographic matrices of this activity correlated well with the published profiles of the TFIIH, BTF2 or δ factors (Conaway and Conaway, 1989a; Gerard *et al.*, 1991; Flores *et al.*, 1992). Thirdly, the ATP analog AMP-PNP, which does not support transcription of linear templates (Figure 5C), is unable to stimulate the DNA helicase activity of TFIIH (Schaeffer *et al.*, 1993). Further support comes from the fact that two of the research groups which identified TFIIH as essential for transcription of the ML promoter used linear templates for their reconstituted reactions (Conaway and Conaway, 1989a; Gerard *et al.*, 1991). Reinberg and co-workers used supercoiled templates and in their experiments a low level of transcription was detected in the absence of added TFIIH (Flores *et al.*, 1992). Clearly, contamination of activities that alter DNA topology (like topoisomerases) in preparations of basal factors or of pol II would render the transcription of a supercoiled template more dependent on TFIIH. In this respect it is important to mention that the topology of the template DNA after incubation in the standard transcription mixture was assessed by agarose gel electrophoresis. No change in the superhelicity of the template DNA could be observed (data not shown).

The transcription factor TFIIH is the only basal factor with known ATP-requiring activities (Conaway and Conaway, 1989b; Lu *et al.*, 1992; Serizawa *et al.*, 1992). It was recently shown that the CTD kinase activity is not essential for transcription (Serizawa *et al.*, 1993). The availability of pol II transcription assays which do not require TFIIH allowed the investigation of the long-standing question why transcription initiation by pol II requires hydrolysis of the β−γ bond of ATP (Bunick *et al.*, 1982). The experimental results shown in Figures 5 and 6 answer this question. The ATP hydrolysis at the β−γ phosphoanhydride bond is required when the DNA helicase activity of TFIIH is required. Negatively-supercoiled templates are efficiently transcribed by pol II using as a substrate the ATP analog AMP-PNP which contains a non-hydrolyzable β−γ phosphoanhydride bond. ATP is a more efficient substrate for transcription by pol II than AMP-PNP. For half-maximal

transcription ~10 μM ATP suffices, whereas ~150 μM AMP-PNP is required to achieve similar transcription levels. ATPγS is as efficient as ATP and AMP-PCP is less efficient than AMP-PNP in supporting pol II transcription (data not shown). This suggests that an intact β−γ phosphoanhydride bond of ATP is important for the K_m of RNA pol II. Paule and co-workers have recently shown that in transcription by RNA polymerase I AMP-PCP does not serve efficiently as the first nucleotide of an rRNA transcript, whereas AMP-PCP is an efficient substrate for the elongation reaction (Lofquist *et al.*, 1993). Interestingly, the ML RNA also starts with an adenine residue. Furthermore, previous studies have indicated that the elongation reaction of pol II with AMP-PNP is 3-fold less efficient compared with the elongation with ATP as a substrate (Bunick *et al.*, 1982).

The finding that negative supercoils in the DNA template can alleviate the requirement for TFIIH in transcription reactions underscores the significance of the DNA helicase activity of this basal factor. The observation that AMP-PNP is unable to support TFIIH-dependent transcription (Figure 5B and C) is in agreement because this is due to the inability of AMP-PNP to support the DNA helicase activity of TFIIH (Schaeffer *et al.*, 1993). The addition of dATP to the reaction with AMP-PNP rescues the DNA helicase activity (Schaeffer *et al.*, 1993) and TFIIH-dependent transcription is restored (Figure 5B and C). Apparently, the topology of the DNA template dictates the requirement for the DNA helicase activity of TFIIH and thus the dependence of the transcription reaction on TFIIH. Although TFIIH is not essential for transcription reactions with supercoiled templates, addition of this factor leads to a 2- to 8-fold stimulation of transcription in a promoter-dependent manner (Figure 4). This effect may result from the tendency of the different supercoiled promoters to melt at the transcription start site in the absence of TFIIH.

Initiation of transcription from linear or relaxed templates exhibited an absolute dependence on TFIIE, but low levels of transcription from supercoiled DNA could be observed in the absence of this factor (Figures 1 and 2B). These low levels were also found with different preparations of pol II and TFIIF (data not shown). Recently Kadonaga and co-workers also found that TFIIE is not essential for ML transcription, but that TFIIE rather has a stimulatory effect (Tyree *et al.*, 1993). The exact function of TFIIE in the assembly of the initiation complex is not known. Provided that the template is negatively supercoiled, transcription of the IgH promoter (Parvin *et al.*, 1992) does not require the action of this factor. It was proposed that TFIIE allows entry of TFIIH and that association of TFIIE and TFIIH with the transcription initiation complex is cooperative (Flores *et al.*, 1992). This model is supported by the observations that: (i) the omission of TFIIH from reactions that lack TFIIE does not result in lower transcription levels (Figure 4) and (ii) the presence of TFIIH reduces the amount of TFIIE required (Figure 2B). However, TFIIE is highly stimulatory for the TFIIH-independent transcription reaction using supercoiled ML and albumin templates (Figures 2B and 4). Therefore, TFIIE must have an additional function independent of TFIIH in the initiation complex assembly on certain promoters. Interestingly, the presence of negative supercoils in the template is also required to allow the low level of transcription in the absence of TFIIE (Figures 2B and 3). It was argued that negative superhelical turns in the TFIIE-independent IgH promoter template provide the energy for melting of the DNA and for formation of the open template conformation (Parvin and Sharp, 1993). It is possible that TFIIE is required for the transcription of promoters that are less liable to form the open complex than the IgH promoter. Open complex formation on the ML promoter, as analyzed by phenanthroline-copper sensitivity, required a fraction that contained TFIIE, TFIIF and probably TFIIH, but surprisingly was independent of ATP (Buratowski *et al.*, 1991). In contrast to this, Gralla and co-workers reported that ATP or dATP was required for the open complex conformation, as determined by potassium permanganate footprinting, which is selective for single-stranded thymine residues. AMP-PNP was inactive in this reaction (Wang *et al.*, 1992). The function of TFIIE could be to stabilize the open template conformation. On templates that do not contain sufficient negative superhelical density, TFIIE alone would be unable to initiate the open complex conformation and requires the assistance of TFIIH to stimulate DNA helicase activity. We are currently testing the sensitivity of different core promoters to potassium permanganate and the effects of the basal factors and of dATP on the formation of the open complex.

The effect of supercoiling of the template on initiation by bacterial RNA polymerase has been well characterized (discussed in Parvin and Sharp, 1993). Several studies in eukaryotic systems have also provided evidence that negative supercoiling is related to activation for transcription (for examples see Leonard and Patient, 1991; Schultz *et al.*, 1992). The model that superhelical density of certain DNA templates determines the requirement for TFIIH, and to a lesser extent for TFIIE, has important implications for transcription regulation of genes. When the activity of these two basal transcription factors is limiting *in vivo*, activation of specific genes (like for example the IgH gene) can be accomplished by an increase in the negative superhelical density at the transcription initiation site. The majority of eukaryotic chromatin is not significantly torsionally strained (Sinden *et al.*, 1980). However, several processes, like for example nucleosome clearance, can account for an increase in negative superhelicity of DNA at specific initiation sequences. This type of regulation has several attractive features. Transcription activation will be promoter-selective because it is not only determined by the topological state but also by the specific DNA sequence of the core promoter. Conversely, transcriptional repression of specific genes could be achieved by removing negative supercoils from the promoter region.

Materials and methods

Materials

Adenylyl-imidodiphosphate, adenylyl (β,γ-methylene)-diphosphate and adenosine-5′-*O*-(3-thiotriphosphate) were obtained from Fluka and Boehringer Mannheim. The AMP-PNP preparation (Figures 5 and 6) was purchased from Boehringer Mannheim and contains <0.1% ATP. Other nucleotides were of the ultrapure grade and were obtained from Pharmacia/LKB. Radiolabeled [α-^{32}P]CTP (760 Ci/mmol) was purchased from Amersham. RNAguard was obtained from Pharmacia/LKB. HEPES, H-8 kinase inhibitor, bovine serum albumin (BSA) and protease inhibitors were purchased from Sigma. Ultrapure ammonium sulfate was acquired from BDH. Restriction endonucleases and dithiothreitol (DTT) were procured from Boehringer Mannheim. Wheat germ topoisomerase I was purchased from Promega. Phosphocellulose P11 was obtained from Whatman. All other chromatography media were acquired from Pharmacia/LKB. Peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

Purification of transcription factors

Recombinant human TBP carrying six histidine residues at its N-terminus was expressed in *Escherichia coli* strain BL21(DE3) and purified using a NiNTA-agarose matrix as described (Parvin *et al.*, 1992). The resulting hTBP fractions contained 30–50% full-length TBP as judged from Coomassie staining of SDS–polyacrylamide gels. The majority of the contaminating proteins was partially degraded TBP which could still bind to the NiNTA matrix.

Protein fraction (AB), containing the basal transcription factors TFIIA and TFIIJ, was obtained from HeLa cell extracts as described previously (Samuels *et al.*, 1982).

Recombinant TFIIB was expressed in *E.coli* strain BL21 carrying the pET-IIB and pLysS plasmids and purified by phosphocellulose chromatography as described (Ha *et al.*, 1991). The TFIIB-containing phosphocellulose fractions were dialyzed with buffer A [20 mM HEPES–KOH pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and 0.1 M KCl, and loaded on a MonoS HR5/5 FPLC column (Pharmacia). The column was washed extensively with buffer A/0.1 M KCl and buffer A/0.2 M KCl and subsequently developed with a linear gradient from 0.2 to 0.6 M KCl in buffer A. Recombinant TFIIB (1.5 mg/ml) eluted in a single peak at 0.32–0.35 M KCl and was homogeneous as judged from Coomassie staining of SDS–polyacrylamide gels.

The 34 and 56 kDa subunits of TFIIE were expressed separately in *E.coli* as described (Peterson *et al.*, 1991). The 34 kDa subunit (34E) was purified from bacterial lysates by chromatography on an S-Sepharose FF column as described (Peterson *et al.*, 1991). Analysis on SDS–polyacrylamide gels of the resulting fractions indicated that most of the 34E protein eluted between the 0.3 and 1 M KCl steps. These fractions were pooled, dialyzed against buffer A/0.1 M KCl, and loaded on a MonoS FPLC column (HR10/10). The bound protein was eluted with a linear gradient from 0.1 to 0.6 M KCl in buffer A. The 34E protein eluted at 0.39 M KCl and was ~90% pure at this stage.

The 56 kDa subunit of TFIIE (56E) was precipitated from bacterial lysates by the addition of solid $(NH_4)_2SO_4$ to 35% saturation. This procedure precipitates only 17% of the total protein while precipitating almost all of 56E (see also Pogonec *et al.*, 1991). Precipitated proteins were dissolved and dialyzed against buffer A/0.15 M KCl. This fraction was loaded onto a DEAE–Sephacel column. After extensive washing, bound proteins were recovered by step elution with buffer A/0.3 M KCl and with buffer A/0.6 M KCl. The majority of 56E elutes in the 0.3 M KCl step. This fraction was adjusted to buffer A/0.2 M KCl and applied to a MonoQ FPLC column (HR10/10). The column was developed with a linear gradient from 0.2 to 0.7 M KCl. Fractions containing 56E protein eluted between 0.35 and 0.45 M KCl. The 56E protein was ~65% pure at this stage.

The TFIIE complex was reconstituted by mixing equimolar amounts of the purified 34E and 56E proteins and dialysis against buffer A/0.1 M KCl for 4 h at 4°C. The dialysate was applied to a MonoS HR5/5 FPLC column and this column was developed with a linear gradient from 0.1 to 0.5 M KCl. TFIIE complex formed efficiently by this procedure (>90%) and separated readily from impurities present in the 56E fraction, which did not stick to the MonoS column, and from the uncomplexed 34E and 56E subunits. Reconstituted recombinant TFIIE eluted as a single peak at 0.22 M KCl and was homogeneous as judged by Coomassie staining of SDS–polyacrylamide gels.

TFIIF was purified in a similar manner to the purification protocol of Conaway and Conaway for the $\beta\gamma$ factor (Conaway and Conaway, 1989a). TFIIF-containing fractions were identified in a standard transcription assay lacking TFIIF and in an immunoblot assay using a polyclonal antiserum directed against RAP30. Purification began with a HeLa cell cytosolic fraction, a side fraction in the preparation of nuclear extract (Challberg and Kelly, 1979). Solid $(NH_4)_2SO_4$ was added to the cytosolic extract (2.4 g of protein) to 40% saturation and precipitated proteins were removed by centrifugation (20 000 *g* for 30 min). Solid $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation. Precipitated proteins (492 mg) were collected by centrifugation, dissolved and dialyzed against buffer A/0.1 M KCl. This fraction was applied to an 80 ml phosphocellulose column. The bound protein was eluted by step elution with buffer A/0.3 M KCl and buffer A/0.6 M KCl. The majority of RAP30 elutes in the 0.6 M KCl step. This fraction (25 mg of protein) was adjusted to buffer A/0.1 M KCl and applied to a 10 ml DEAE–Sephacel column. Bound proteins were eluted by step elution with buffer A/0.3 M KCl and with buffer A/0.6 M KCl. The 0.3 M KCl fraction (14.7 mg of protein) contained the majority of RAP30. From this step, 1 μg/ml aprotinin and 1 μg/ml pepstatin were added to all buffers and the concentration of DTT was increased to 2 mM. 10 mg of the 0.3 M DEAE fraction was dialyzed against buffer A plus 1.5 M $(NH_4)_2SO_4$, and applied to a phenyl-Superose FPLC column (HR5/5; Pharmacia). The column was developed with a linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$. TFIIF-containing fractions eluted at 0.80–0.65 M $(NH_4)_2SO_4$ and were dialyzed against buffer A/0.1 M KCl and applied to a MonoS HR5/5 FPLC column. Bound proteins were eluted with a linear gradient from 0.1 to 0.5 M KCl in buffer A. TFIIF-containing fractions eluted at 0.18–0.26 M KCl, were adjusted to buffer A/0.1 M KCl and applied to a MonoQ HR5/5 FPLC column. The column was developed with a linear gradient from 0.1 to 0.6 M KCl. Fractions that contained RAP30 (0.22–0.26 M KCl) were pooled and used as TFIIF (45 μg of protein per ml) in transcription reactions.

Purification of TFIIH was monitored by immunoblot analysis using the M.Ab.3C9 mAb directed against the 62 kDa subunit of TFIIH (62H) (Fischer *et al.*, 1992) and by stimulation of the linear transcription reaction. Purification began with the 0.3–0.6 M KCl phosphocellulose fraction prepared as described (Samuels *et al.*, 1982). This fraction (52 mg of protein) was adjusted to buffer A/0.1 M KCl and applied to a 10 ml DEAE–Sepharose FF column. After extensive washing the column was developed by a step elution with buffer A/0.3 M KCl and with buffer A/0.6 M KCl. The majority of the 62H protein eluted in the 0.3 M KCl step. From this step, 1 μg/ml aprotinin and 1 μg/ml pepstatin were added to all buffers and the concentration of DTT was increased to 2 mM. The 0.3 M DEAE fraction (20 mg of protein) was dialyzed against buffer T (20 mM Tris–HCl pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) plus 0.1 M KCl and loaded on a MonoQ HR5/5 FPLC column. The column was developed with a linear gradient from 0.1 to 0.6 M KCl in buffer T and the 62H protein eluted at 190–280 mM KCl. TFIIH-containing fractions (3.6 mg of protein) were pooled, dialyzed against buffer A/1 M $(NH_4)_2SO_4$ and applied to a phenyl-Superose HR5/5 FPLC column. The column was eluted with a linear gradient of 1.0 to 0.0 M $(NH_4)_2SO_4$ in buffer A. The 62H protein eluted between 0.45 and 0.15 M $(NH_4)_2SO_4$. These fractions (1.55 mg of protein) were pooled, dialyzed against buffer A/0.1 M KCl and applied to a MonoQ HR5/5 column. The column was eluted with a linear gradient from 0.1 to 0.6 M KCl. TFIIH peak fractions (290 μg of protein per ml) eluting at 0.24 M KCl were pooled and used as TFIIH in standard transcription reactions.

The RNA pol II used was an amanitin-resistant polymerase from the Ama1 CHO cell line. This preparation was estimated to be >90% pure as judged by silver-staining of SDS–polyacrylamide gels (Cartew *et al.*, 1988).

In vitro transcription reactions and DNA templates

Transcription reactions were assembled on ice and contained 12 mM HEPES–KOH (pH 7.9), 60 mM KCl, 12% glycerol, 0.6 mM EDTA, 0.3 mM PMSF, 1.2 mM DTT, 5–7.5 mM $MgCl_2$, 30–60 μg/ml BSA and 10 U RNAguard. Unless indicated in the figure legends, transcription reactions also contained 5 μg/ml pML(C_2AT)19δ-51 (Sawadogo and Roeder, 1985), 60 μM ATP, 60 μM UTP, 10 μM [α-^{32}P]CTP (50 Ci/mmol) and 60 μM 3′-OMe-GTP. All transcription factor preparations were adjusted to buffer A/0.1 M KCl containing 0.1 mg/ml BSA to stabilize proteins. Except when titrated, the amount of basal transcription factor per standard reaction was 100 ng bacterially-expressed His-TBP, 50 ng bacterially-expressed TFIIB, 50 ng bacterially-expressed TFIIE, 2 μl partially-purified TFIIF (45 μg/ml), 1.8 μl partially-purified TFIIH (290 μg/ml) and 0.25 μl RNA pol II in a 20 μl reaction volume.

Reaction mixtures were incubated for 45–60 min at 30°C. Reactions were stopped and processed as described (Timmers and Sharp, 1991). Radioactive RNA products were quantitated using a PhosphoImager gel scanner (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.25 software.

Other transcription templates used were: pMLC_2ATδ-71 (Szentirmay and Sawadogo, 1991) containing the adenovirus major-late promoter sequences −71 to +10 driving a 271 nt guanosine-less cassette (kind gift from Dr M.Sawadogo, University of Texas, Houston, TX); pμ(−47)-G- (Parvin *et al.*, 1992) containing the IgH core promoter driving the 370 nt guanosine-less cassette (kindly provided by J.Parvin, MIT, Cambridge, MA); pL-TG containing the *Xenopus* albumin core promoter (−67 to −22, kindly provided by P.Herrlich, Karlsruhe, Germany) driving a 370 nt guanosine-less cassette (Ryffel *et al.*, 1989); and pMV37 containing the MMTV core promoter (−37 to +10) driving a 380 nt guanosine-less cassette (manuscript in preparation).

Supercoiled transcription templates were prepared as described (Timmers and Sharp, 1991) and linear pML(C_2AT)19δ-51 was prepared by *Eco*RI digestion. Relaxed pML(C_2AT)19δ-51 template was prepared by incubation with wheat germ topoisomerase I according to the manufacturer's specifications. Topoisomerase I was removed from the relaxed DNA template by phenol/chloroform extraction and ethanol precipitation. Analysis of the superhelical density of the resulting plasmid DNA by agarose gel electrophoresis indicated that the DNA was covalently closed and that it contained on average four superhelical turns per template molecule.

Immunoblot analysis

Protein samples were separated in 12.5% SDS–polyacrylamide gels and blotted onto BA85 nitrocellulose (Schleicher and Schull) as described (Timmers and Sharp, 1991). Protein blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20. Blots were probed with a 1:8000 dilution of M.Ab.3C9 ascites fluid (kind gift from Drs Roy and Egly, INSERM, Strasbourg), a 1:1000 dilution of a polyclonal rabbit antiserum specific for the 56 kDa subunit of TFIIE, or a 1:500 dilution of a polyclonal rabbit antiserum specific for the RAP30 subunit of TFIIF (kind gifts from Dr J.Parvin, MIT, Cambridge, MA). Immunoblots were developed using the appropriate peroxidase-conjugated antibodies and the ECL detection kit from Amersham according to the manufacturer's specifications.

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