Transcription factor TFIIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance

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The basal factor TFIIE is an important component of the RNA polymerase II transcription machinery. In our efforts to determine the role of TFIIE in the transcription process, we have begun to define the interactions between TFIIE and other basal transcription factors. Here we report that TFIIE binds selectively to the nonphosphorylated form of RNA polymerase II (IIa) and that this interaction is mediated by the 56-kD subunit of TFIIE. Additional binding studies reveal that TFIIE can interact with TBP as well as TFIIID and that this interaction is mediated primarily via the 56-kD subunit. Our studies indicate that TFIIE also interacts with both subunits of TFIIF and with TFIIH, a multisubunit basal factor reported to catalyze RNA polymerase II CTD phosphorylation. Protein affinity assays demonstrate that TFIIE binds directly to ERCC-3, a DNA repair protein associated with TFIIH. More importantly, TFIIE affinity resin can selectively isolate transcriptionally competent TFIIH from a partially purified preparation and thereby may recruit TFIIH to the transcription complex *in vivo*. These multiple interactions between TFIIE, RNA Pol II and TFIIH support a model in which TFIIE plays a role in promoter clearance as well as in the recruitment of proteins required for transcription-coupled DNA repair.

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Transcription of protein-encoding genes in eukaryotes is directed by RNA polymerase II (RNA Pol II) and a complement of auxiliary factors. One class of factors is comprised of sequence-specific DNA-binding proteins that recognize unique promoter elements and subsequently regulate transcription (for review, see Mitchell and Tjian 1989). A second class of factors, the basal transcription factors, exerts its effects through the core elements of RNA Pol II promoters (TATA box and initiator motif), and is required for accurate initiation and transcription at most, if not all, promoters (for review, see Zawel and Reinberg 1993). Although the basal factors (TFIIA, TFIIB, TFIIID, TFIIE, TFIIF, and TFIIH), have been identified based on their ability to reconstitute transcription *in vitro*, the mechanism by which many of the individual factors contributes to the formation of an active transcription complex remains unclear. For example, it is unlikely that all of these ancillary basal factors are required for the initiation of transcription. Instead, it is likely that some of them direct initiation while others act during the promoter clearance or elongation steps of the reaction. Additionally, any and all of these defined steps of transcription may be targets for specific regulatory factors that could serve to accelerate or inhibit the

various steps of transcription directly. The recent availability of purified recombinant basal factors provided us with an opportunity to carry out a more rigorous analysis of the activities and interactions of the basal factors with other members of the transcription complex, thereby providing a clearer understanding of the events that govern mRNA synthesis.

Previous work had shown that human TFIIE is a heterotetramer comprised of two subunits of 34 and 56 kD (TFIIE-34 and TFIIE-56; Inostroza et al. 1991). The subunits of TFIIE are in a 1:1 ratio in the heterotetramer and appear to be required in the same ratio for optimal reconstituted transcription and entry into a partially formed transcription complex as measured by gel mobility-shift analysis (Peterson et al. 1991). Although TFIIE has been considered one of the basal or general factors required to initiate transcription at all RNA Pol II promoters, several studies suggest that at least some promoters can be transcribed without TFIIE (Parvin et al. 1992; Tyree et al. 1993). In addition, recent abortive initiation assays that are able to discriminate between factors required for initiation versus promoter clearance and elongation suggest that TFIIE may not be required for the initiation step at some promoters (J. Goodrich, in

prep.). Similarly, studies of elongation complexes suggest that TFIIE is released from the transcription complex once the elongation phase of transcription is reached (D. Reinberg, pers. comm.). The role of TFIIE is therefore of considerable interest because it does not appear to be required for either initiation or elongation, and yet it is clear from the available data that it is an essential transcription factor for most templates that have been tested.

In this report, we have attempted to determine the role of TFIIE during transcription. In particular, we have assayed the ability of TFIIE subunits to make protein-protein contacts with all of the known components of the basal transcriptional machinery. Interestingly, the two subunits of TFIIE interact selectively with multiple targets and our findings suggest a model in which TFIIE plays a role in TFIIF recruitment, transcription complex stabilization, promoter clearance, and association of ancillary enzymatic complexes involved in DNA repair.

Results

TFIIE interacts with the nonphosphorylated form of RNA Pol II

Assembly of the preinitiation complex, as shown by gel mobility shift analysis (Buratowski et al. 1989; Flores et al. 1991), is a highly ordered process that is thought to begin with the recognition of promoter elements by TFIID, which consists of the TATA-binding protein (TBP) and at least eight associated TAFs (TBP-associated factors) (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1992). The binding of TFIIB and TFIIF in turn stabilizes the TFIID/DNA complex (Buratowski et al. 1989; Maldonado et al. 1990). It is this structure that is recognized by the TFIIF/RNA Pol II complex (Flores et al. 1991). TFIIE is then thought to enter the transcription assembly, and subsequently, TFIIF and TFIIE associate to form a transcription competent complex (Inostroza et al. 1991; Flores et al. 1992). Because the entry of TFIIE into the preinitiation complex is preceded by several other factors, there exist many potential interaction surfaces that may participate in the union of TFIIE with the preinitiation superstructure. The components of the complex that enter immediately prior to TFIIE are TFIIF and RNA Pol II, and these are thought to enter together. In light of previous studies that showed a cosedimentation of TFIIE and RNA Pol II (Reinberg and Roeder 1987a; Flores et al. 1989), we first asked whether TFIIE could recognize and bind RNA Pol II to form a stable complex in solution. Our experimental strategy was designed to also identify which subunit(s) of TFIIE contacted RNA Pol II and which form of RNA Pol II would be associated with TFIIE.

RNA Pol II can be purified from HeLa cells in both its phosphorylated (Ilo) and nonphosphorylated (IIa) forms (Reinberg and Roeder 1987a; Lu et al. 1991). The Ilo form of the enzyme possesses a multitude of phosphorylated residues at the carboxyl terminus of its largest subunit,

which consists of 52 heptapeptide repeats (Corden et al. 1985). Extensive phosphorylation of this carboxy-terminal domain (CTD) results in a decreased mobility of the largest subunit (240 kD) with respect to the nonphosphorylated large subunit (200 kD). The RNA Pol II purified from HeLa cells used in our experiments is a mixture of the Ilo and IIa forms of the enzyme as shown in lanes 1 and 2 of Figure 1. To detect direct binding between TFIIE and RNA Pol II, we have carried out coimmunoprecipitation experiments with recombinant TFIIE subunits. The large subunit of TFIIE and the nonphosphorylated IIa form of RNA Pol II are efficiently coimmunoprecipitated with antibodies against TFIIE-56 either in the presence or absence of TFIIE-34 (Fig. 1, lanes 4 and 5, respectively), with more RNA Pol II being coimmunoprecipitated in the presence of TFIIE-34. In striking contrast, we found very little of the phosphorylated Ilo form of RNA Pol II coprecipitated with TFIIE-56 even though the starting material contains a molar excess of the phosphorylated form of the enzyme. In a control experiment to demonstrate that the Ilo form had not been inadvertently converted to the IIa form by contaminating phosphatases in our protein preparations, we assayed the supernatants of the immunoaffinity reactions and found that the phosphorylated species was present and intact (Fig. 1, lanes 6–8) and that the nonphosphorylated species had been significantly depleted (Fig. 1, lane 7). These results indicate that the 56-kD subunit of TFIIE can interact directly and selectively with the IIa form of RNA Pol II. This finding is consistent with previous studies demonstrating that it is primarily the nonphosphorylated form of RNA Pol II that is recruited to the transcription complex at the promoter (Laybourn and Dahmus 1990; Lu et al. 1991). In similar experiments using exclusively RNA Pol Ilo as the input, a very small amount of the phosphory-

Figure 1. TFIIE preferentially binds the nonphosphorylated form of RNA Pol II. A mixture of RNA Pol Ilo and RNA Pol IIa (lanes 1, 2) was used in coimmunoprecipitation experiments. The binding of RNA Pol II to TFIIE was analyzed using anti-TFIIE-56 antiserum alone (lane 3) and in the presence of the TFIIE subunits: TFIIE-56 (lane 4) TFIIE-56, and TFIIE-34 (lane 5). The corresponding supernatants of the immunoprecipitations are shown in lanes 6, 7, and 8 respectively. The presence of the large subunit of RNA Pol II was detected by Western blot using a monoclonal antibody specific for the carboxy-terminal domain.

lated form of the enzyme can be coimmunoprecipitated (data not shown), suggesting that in the absence of RNA Pol II, TFIIE can weakly recognize RNA Pol II consistent with the observation that RNA Pol II can be inefficiently assembled into the transcription complex (Lu et al. 1991). Our results suggest that in addition to preferentially recognizing the nonphosphorylated form of RNA Pol II, TFIIE may as a consequence be routinely recruited to the preinitiation complex even though it may not be required until after the initiation of the first phosphodiester bond.

TFIIE interacts with TFIIF

Because TFIIF is thought to recruit RNA Pol II to the promoter (Flores et al. 1991) and enters the transcription complex with RNA Pol II, we were interested in determining whether TFIIE can also interact directly with TFIIF. We generated two specific affinity resins using polyclonal antibodies against the subunits of TFIIE and protein A-Sepharose beads. Both resins nonspecifically retain a small amount of the TFIIF subunits, RAP30 and RAP74, in the absence of TFIIE subunits (Fig. 2, lanes 1 and 2 for α -TFIIE-56 resin and lane 9 for α -TFIIE-34 resin). The addition of purified recombinant TFIIE-56 to α -TFIIE-56 resin results in little or no binding of RAP30 above the background level (Fig. 2, lane 3), but leads to a

Figure 2. TFIIE interacts with TFIIF. Radiolabeled subunits of TFIIF, *RAP30, and *RAP74 were tested for their ability to be coimmunoprecipitated with TFIIE subunits. Lanes 7 and 8 show the input proteins *RAP30 and *RAP74, respectively. Proteins were tested for nonspecific binding on control resins consisting of protein A-Sepharose and α -TFIIE-56 antiserum (lanes 1,2) and α -TFIIE-3 antiserum (lane 9) in the absence of added recombinant TFIIE subunits. The addition of TFIIE-56 to α -TFIIE-56 immuno-affinity resin results in little or no specific retention of *RAP30 (lane 3) but results in a significant retention of *RAP74 (lane 4). The addition of TFIIE-34 in the presence of TFIIE-56 results in an even greater amount of *RAP74 (lane 5) and *RAP30 (lane 6) retained by α -TFIIE-56 immuno-affinity resin. In the presence of TFIIE-34, α -TFIIE-34 immuno-affinity resin retains no *RAP74 above background (lane 10) but specifically retains a significant level of *RAP30 above background (lane 11).

modest but significant amount of specific RAP74 binding (Fig. 2, lane 4). The addition of purified recombinant TFIIE-34 to the TFIIE-56 resin results in a marked increase in the amounts of RAP74 (Fig. 2, lane 5) and RAP30 (Fig. 2, lane 6) bound by the resin relative to background levels (Fig. 2, lanes 2 and 1, respectively). A similar analysis with α -TFIIE-34 resin results in a specific retention of RAP30 (Fig. 2, lane 11) above nonspecific binding (Fig. 2, lane 9), although there is little or no specific binding of RAP74 (Fig. 2, lane 10) by TFIIE-34 above background (Fig. 2, lane 9). Interestingly, it appears that under the conditions of our assay, RAP30 is the subunit of TFIIF bound preferentially by TFIIE-34 (Fig. 2, lane 11) and RAP74 is the subunit bound preferentially by TFIIE-56 (Fig. 2, lane 4). Furthermore, TFIIE-56 specifically retains an increased amount of RAP74 in the presence of TFIIE-34, suggesting that TFIIE-34 is required for stable interactions between TFIIE and TFIIF. In light of our observation that TFIIE-56 can stably associate with RNA Pol II in the absence of TFIIE-34, it is possible that TFIIE-34-TFIIF interactions function to help stabilize the complex. This may explain the observation that TFIIE-56-RNA Pol II interactions are not sufficient for the formation of a stable transcription complex during gel mobility-shift assays (Peterson et al. 1991); the requirement for an equimolar amount of TFIIE-34 suggests that TFIIE-34-mediated binding of TFIIF may contribute to the stable association of the TFIIE heterotetramer with the transcription complex.

TFIIE interacts with TBP

Given the selective interaction of TFIIE with the nonphosphorylated form of RNA Pol II, and earlier studies that report a direct interaction between TBP and the nonphosphorylated CTD (Usheva et al. 1992), we considered the possibility that TFIIE may also interact with TBP. To test this idea, we generated a resin containing an immobilized glutathione-S-transferase-TFIIE-56 (GST-56) fusion protein and analyzed the ability of this protein-affinity resin to specifically retain [35 S]methionine-labeled in vitro-translated TBP. Several other subunits of TFIID including dTAF_{II}30 α and dTAF_{II}30 β , as well as the largest subunit of the basal transcription factor TFIIA were also tested as controls for specificity of binding. Under the conditions of our assay, TBP was the only protein specifically retained by GST-56 beads (Fig. 3A, lane 8). In contrast, TFIIA, dTAF_{II}30 α , and dTAF_{II}30 β (lanes 6 and 7, respectively), recombinant proteins shown to bind other partners in similar assays (Yokomori et al. 1993a,b), failed to bind, suggesting that the observed interaction between TBP and TFIIE-56 is specific.

If the interaction between in vitro-translated radiolabeled TBP and GST-56 is significant, we would expect that TBP-TFIIE-56 binding should also occur when purified recombinant TBP is tested. As predicted, GST-56 affinity resin bound TBP (Fig. 3B, lane 3) whereas GST control resin failed to bind TBP (Fig. 3B, lane 2). In further experiments, not shown here, several additional af-

Figure 3. TFIIE-56 interacts with TBP and TFIID. (A) Radiolabeled RNA Pol II-specific transcription factors were tested for their ability to bind to a GST-56 fusion protein immobilized on glutathione beads in 0.15 M KCl binding buffer. Lanes 1–3 show the input of TBP, dTAF_{II}30 β , dTAF_{II}30 α , and the large subunit of TFIIA. Proteins were tested for nonspecific binding on a control GST resin (lanes 4, 5; proteins tested as indicated) and for TFIIE-56-specific binding on GST-56 resin (lanes 6–8) as indicated. (B) Bacterially expressed purified human TBP was assayed for its ability to bind GST-56 resin in 0.1 M KCl binding buffer, and the bound material was visualized by Western blot analysis using a monoclonal antibody to TBP. Lane 1 shows the TBP input. TBP is specifically retained by GST-56 resin (lane 3) but not by GST control resin (lane 2). (C) A partially purified TFIID fraction was tested for the ability to bind GST-56 resin in 0.1 M KCl binding buffer, and the bound material was visualized using a cocktail of antibodies specific for TBP, hTAF_{II}80, and hTAF_{II}250. Lane 1 shows the input TFIID fraction. GST-56 specifically retains TBP, hTAF_{II}80, and hTAF_{II}250 (lane 3) but control GST resin does not (lane 2).

finity resins, including GST–TBP, GST–carboxy-terminal TBP, and TFIIE-56 immuno-affinity resins, were generated to analyze TFIIE-56–TBP binding. In all cases, stable TFIIE-56–TBP binding was observed, supporting the notion that TFIIE-56 and TBP interact, and do so via the conserved carboxy-terminal domain of TBP.

Because it is likely that TBP seldom exists as a single subunit *in vivo*, we were also interested in determining whether TFIIE-56 could interact with the native TFIID complex. Using GST-56 resin and a phosphocellulose fraction containing native TFIID, we found that the GST-56 resin was competent for binding to the TFIID complex, as determined by Western blotting using a cocktail of antibodies that recognizes TBP, as well as two other subunits of TFIID, hTAF_{II}80 and hTAF_{II}250 (Fig. 3C, lane 3). This result not only demonstrates that TFIIE can bind native TFIID, but also that this interaction results in a TFIIE–TFIID complex that is stable for *in vitro* isolation. These findings, taken together with the RNA Pol II data, indicate that TFIIE participates in interactions with both RNA Pol II and with TBP, a protein known also to bind to the nonphosphorylated CTD of RNA Pol II. It therefore became of interest to determine whether TFIIE could also interact directly with and thereby recruit TFIIF, a factor known to bind after TFIIE complex assembly (Flores et al. 1992) and reported to possess RNA Pol II CTD kinase activity (Lu et al. 1992).

TFIIE interacts with TFIIF and ERCC-3

The multisubunit TFIIF fraction has been shown to phosphorylate the CTD of RNA Pol II, and TFIIE can

stimulate the kinase activity of TFIIF (Lu et al. 1992). We therefore examined whether or not TFIIE could bind stably to TFIIF. GST-56 resin was prebound with TFIIE-34 to generate a matrix containing both subunits of TFIIE. Using a partially purified fraction that contains TFIIF transcriptional activity, we tested the ability of the TFIIE resin to interact with TFIIF. We identified the bound species by Western blot analysis using antibodies to the cloned 62-kD subunit of TFIIF, p62 (Fischer et al. 1992). As shown in Figure 4, TFIIE resin retains an increasing amount of TFIIF p62 as the amount of TFIIF fraction is increased (lanes 3–5), and there is a corresponding depletion of TFIIF p62 from the supernatant. These data suggest that TFIIE can bind specifically and directly to one or more subunits of TFIIF.

Recently, ERCC-3, a protein known to be involved in DNA repair (Weeda et al. 1990), has been reported to be a subunit of the TFIIF complex (Schaeffer et al. 1993). We examined whether or not TFIIE stably interacts with ERCC-3 using a maltose-binding protein (MBP)–ERCC-3 fusion product linked to amylose beads (MBP–ERCC-3 resin). We found that the MBP–ERCC-3 resin could retain both the large and small subunits of TFIIE (Fig. 5, lanes 4–6), whereas control MBP could not (Fig. 5, lane 3). In addition, MBP–ERCC-3 resin does not retain a control protein, TFIIA (Fig. 5, lane 9), suggesting that the interaction of TFIIE with ERCC-3 is specific. These results indicate that TFIIE interacts with ERCC-3 through protein–protein contacts.

Having established that TFIIE can interact with TFIIF, we then tested the ability of TFIIE to recruit the intact multiprotein TFIIF complex. Specifically, we asked

Figure 4. TFIIE Interacts with TFIIH. A partially purified TFIIH preparation (lane 1) was tested for the ability to bind GST-56 resin in 0.1 M binding buffer and was analyzed by Western blot using antibodies against the p62 subunit of TFIIH. Incubation of the partially purified TFIIH fraction with GST control resin resulted in no retention of p62 in the beads (lane 2). Instead, p62 remained in the supernatant in its intact form (lane 6). In contrast, GST-56 resin efficiently retained p62 (lanes 3–5) such that a corresponding depletion of the supernatant was observed (lanes 7–9).

whether one of the TFIIE subunits could affinity isolate functional TFIIH. A partially purified TFIIH preparation was applied to the GST-56 affinity resin under relatively

Figure 5. TFIIE interacts with ERCC-3. Radioactive TFIIE subunits were tested for the ability to bind a MBP-ERCC-3 fusion protein immobilized on amylose beads and in 0.1 M binding buffer. Lanes 1 and 2 show input TFIIE-56 and TFIIE-34, respectively. Arrows indicate positions of full-length products. Control MBP beads did not retain either subunit of TFIIE (lane 3). In contrast, MBP-ERCC-3 resin retained both the TFIIE-56 and TFIIE-34 subunits of TFIIE when they were tested independently (lanes 6 and 4, respectively) or together (lane 5). Radioactive TFIIE large subunit, a protein known to bind other proteins in similar assays (Yokomori et al. 1993a), did not bind to either the control MBP or MBP/ERCC-3 resins (lanes 8 and 9, respectively).

low ionic conditions. After repeated washing to remove unbound material, we eluted the beads with high salt. We observed TFIIE p62 in the 0.5 M eluate from TFIIE resin (Fig. 6A, lane 4) but not from control GST resin (Fig. 6A, lane 2). Importantly, activity eluted from the GST-56 resin was able to restore transcriptional activity to a reconstituted reaction dependent on added TFIIE (Fig. 6B, lanes 9,10). Material eluted from the control resin had no TFIIE activity (Fig. 6B, lanes 5,6). The reconstitution of transcription by the fraction eluted from the GST-56 resin suggests that the bound material contains an active form of TFIIE and that the interactions between TFIIE and TFIIH observed *in vitro* may reflect the recruitment of TFIIE to the transcription complex by TFIIE.

Discussion

Our efforts to define the role of TFIIE in transcription have focused on a systematic analysis of interactions of

Figure 6. TFIIE-56 resin can affinity purify a transcriptionally active TFIIH complex. (A) A partially purified TFIIH fraction was applied to control GST or GST-56 resin under low ionic strength conditions (0.1 M binding buffer) and washed extensively. Western blot analysis using TFIIE p62 antibodies revealed that the 0.5 M KCl elution from GST-56 resin contained TFIIE p62 (lane 4), where an equivalent elution from GST control resin did not (lane 2). (B) The 0.5 M eluate from the GST-56 resin contains TFIIE transcriptional activity. A reconstituted HeLa transcription system that was absolutely dependent on TFIIE (lanes 1,2) was used to test the GST and GST-56 resin eluates for TFIIE activity. The 0.5 M eluate from the GST-56 resin specifically contained TFIIE activity (lanes 9,10), whereas both the 0.5 M and 1.0 M eluates from the control GST resin (lanes 5–8) as well as the 1.0 M eluate from the GST-56 resin (lanes 11,12) did not contain TFIIE activity.

TFIIE with known components of the basal transcription machinery and have revealed that this basal factor participates in several potentially important interactions with many of the components of the RNA Pol II transcription complex. Significantly, TFIIE binds preferentially to the nonphosphorylated form of RNA Pol II, despite the presence of a molar excess of the phosphorylated form. TFIIE also associates with TFIID and binds selectively to the multisubunit TFIIH complex. The interaction between TFIIE and TFIIH is sufficiently specific and robust in vitro to allow the purification of a transcriptionally active TFIIH complex. Additional binding studies reveal that TFIIE participates in protein-protein contacts with TFIIIF, and with ERCC-3, a DNA repair helicase reported to be a subunit of TFIIH [Schaeffer et al. 1993]. These binding studies support the notion that TFIIE is an integral component of the transcription complex responsible for orchestrating or directing multiple interactions, and that these interactions may play a role in recruiting essential transcription factors such as TFIIH. Though these data indicate that specific interactions between components of the transcription complex can occur in vitro, further experimentation is required to assess any functional relevance of these interactions in vivo.

Recent in vitro studies have demonstrated that TFIIE is not required for transcription of certain promoters on supercoiled templates while it is required for transcription from the same promoters on linear templates [Parvin and Sharp 1993]. However, abortive initiation as-

says that detect open promoter complexes capable of cycling trinucleotide transcripts, a hallmark of the initiation step of transcription, have revealed that TFIIE is not required for initiation of transcription even from linear templates [Goodrich and Tjian 1994]. Furthermore, analysis of ternary elongation complexes suggests that TFIIE is not associated with the elongating RNA Pol II complex [D. Reinberg, pers. comm.]. Our efforts to define the function of TFIIE have revealed some intriguing results establishing that TFIIE interacts with multiple components of the transcription machinery. Our results, taken together with recent transcription experiments that dissect some of the discrete steps during transcription (i.e., initiation, promoter clearance, and elongation) suggest a model for the assembly, recruitment and subsequent dynamic alteration that must occur during the transition from initiation to elongation. A model for the potential participation of TFIIE during transcription is shown in Figure 7.

First, the recruitment of TFIIE to the promoter follows the simultaneous association of TFIIIF and the nonphosphorylated form of RNA Pol II (IIa) to the TFIID/TFIIB complex. We envision that TFIIE enters into the transcription complex through multiple interactions with several components already assembled at the promoter including RNA Pol II, TBP, and TFIIIF. One of the primary interactions may be between TFIIE and RNA Pol IIa, the form of the enzyme recruited to the promoter by TFIIIF. The observed selectivity of TFIIE for the (non-phosphorylated) RNA Pol IIa suggests that TFIIE may

Figure 7. A model for the role of TFIIE in the process of promoter clearance. (A) The active initiation complex (for the purposes of simplicity, TFIID, and components of TFIID other than TBP have been omitted). (B) Assembly of TFIIE into the complex via binding of RNA Pol IIa, TBP, and TFIIIF. (C) TFIIE recruits TFIIH and ERCC-3 by direct interactions with both TFIIE-34 and TFIIE-56 subunits. (D) Association of TFIIH with the transcription complex allows for the phosphorylation of the CTD of RNA Pol II. (E) Promoter clearance occurs, releasing TBP, TFIIIF, TFIIE, and TFIIH and triggering the elongation mode of transcription by RNA Pol II.

bind the nonphosphorylated CTD directly as a mechanism for discriminating between the two forms. However, our preliminary studies did not reveal any stable interaction between the CTD and TFIIE subunits (data not shown). Alternatively, TFIIE may interact with other domains or subunits of RNA Pol II that are conformationally sensitive to the phosphorylation state of the CTD. Because the nonphosphorylated RNA Pol II is bound by both TBP and TFIIE, the binding of TFIIE may serve to stabilize the partially assembled transcription complex and also mediate the formation of an interface for TFIIF binding. Further evidence for a role for TFIIE in complex stabilization is provided by preliminary experiments using TFIIE affinity resins showing that TFIIE also interacts with TFIIF. We believe these interactions to be specific and primarily mediated by TFIIE-34, the small subunit of TFIIE (data not shown).

Previous studies have revealed that TFIIF does not bind a partially formed transcription complex in the absence of TFIIE as measured by gel mobility-shift assays (Flores et al. 1992). Our results now establish that TFIIE most likely interacts directly with components of TFIIF and can stably bind to an intact and functional TFIIF complex. These specific and possibly obligatory protein-protein contacts between TFIIE and TFIIF are likely to recruit TFIIF to the transcription complex containing the nonphosphorylated form of RNA Pol II. This recruitment of TFIIF by TFIIE may in turn target TFIIF to the CTD, thus mediating the subsequent phosphorylation of the CTD by the kinase activity associated with TFIIF. The change in the phosphorylation state of RNA Pol II may in turn signal the release of TBP (Usheva et al. 1992; Drapkin et al. 1993), and (because it does not bind the phosphorylated form) TFIIE from RNA Pol II, thus altering the assembly and poising the complex for movement from the open promoter structure into the elongation mode of transcription. Although TFIIF has been shown to phosphorylate the CTD in the absence of the other components of the transcription complex in vitro, kinase activity is greatly stimulated by assembly of a preinitiation complex including TFIIE. More specifically, the addition of TFIIE has been reported to stimulate the kinase activity of TFIIF both qualitatively (conferring the efficient use of [γ - 32 P]ATP as a substrate) and quantitatively (contributing to a fourfold increase in phosphorylation of RNA Pol II) (Lu et al. 1992). Therefore, TFIIE may not only recruit TFIIF, but target it directly to the CTD, and/or induce a conformational change in TFIIF that facilitates its kinase activity. We propose that recruitment of TFIIF by TFIIE is required to enable kinase and/or helicase functions in order to trigger the promoter clearance step of transcription.

Our experiments also reveal an interaction between TFIIE and ERCC-3, a DNA-repair protein reported to be a subunit of TFIIF. Recently, factor b, the yeast equivalent to TFIIF, has been reported to be comprised primarily of DNA repair proteins (Feaver et al. 1993). The association of TFIIE, TFIIF, and DNA repair proteins suggests that these factors may function to couple transcription and DNA repair. Several reports indicate

that the template strand of actively transcribed genes is repaired more efficiently than the nontemplate strand, and considerable data support the proposed coordination of these two processes in both eukaryotes (Bohr et al. 1985; Mellon et al. 1986) and prokaryotes (Mellon and Hanawalt 1989; Selby and Sancar 1993). Although the coupling of transcription and DNA repair remains less clear in eukaryotes, the ERCC-6, ERCC-3, and ERCC-2 gene products have been implicated in the process (Wauthier et al. 1990; Lommel and Hanawalt 1991). Interestingly, the association of ERCC-3 with TFIIE and TFIIF implies a recruitment of such DNA repair proteins during the assembly of the transcription complex.

The association of DNA repair proteins with TFIIE/TFIIF raises the following question: Why are DNA repair components coupled to transcription factors involved in early events during transcription? The interaction of DNA repair proteins with the transcriptional apparatus raises the possibility that TFIIE may participate in two distinct functions. It is possible that like RNA Pol IIa at the the initiation site which requires additional factors (TFIIE and TFIIF) to clear the promoter, RNA Pol II stalled at a DNA lesion may require the reassembly of additional factors to repair the lesion and continue the process of transcription. In this model, the RNA Pol II stalled at a lesion would be recognized by TFIIE which, perhaps via ERCC-3, recruits TFIIF and the associated DNA repair proteins to the lesion. Furthermore, this model proposes that stalled RNA Pol II would exist in the dephosphorylated form, the species that is able to efficiently bind TFIIE. Dephosphorylation of RNA Pol II stalled at a template lesion could perhaps be generated through the action of an associated phosphatase that specifically recognizes a halted RNA Pol II complex. In support of this notion, perhaps, is the observation of an activity in HeLa cell extracts that has been shown to catalyze the dephosphorylation of the CTD (Chesnut et al. 1992), though the substrate specificity and characterization of this activity remain unclear. An example of a condition where re-recruitment of TFIIE/TFIIF may be required apparently exists at the *Drosophila melanogaster* HSP70 promoter, where analysis has revealed that the RNA Pol II halted at the pause site (+20 to +40 relative to the start of transcription), is in the nonphosphorylated state in vivo (T. O'Brien and J. Lis, pers. comm.). Whether the nonphosphorylated RNA Pol II in these experiments is a result of a dephosphorylation event or reflects a condition where RNA Pol II was not initially phosphorylated prior to transcription is not known. Because the available data suggest that in vivo, genes that are actively transcribed are associated specifically with RNA Pol IIo (Weeks et al. 1993) and have not yet been shown to be associated with RNA Pol IIa under any experimental conditions, it is possible that a rephosphorylation event must occur before the stalled complex can continue elongation. A re-recruitment of TFIIE and TFIIF may be required before the halted RNA Pol II, be it at a pause site or a DNA lesion, can resume elongation of the transcript. The formation of an assembled complex containing TFIIE may therefore lead to either initi-

ation or repair, depending on the status of the template bound by RNA Pol II. Assembly of such a "bifunctional" transcription complex provides flexibility in supplying a modular set of factors that can participate in transcription or DNA repair. Although it is currently unknown whether an RNA Pol II complex stalled at a DNA lesion resumes elongation from the stall site, or whether the entire complex dissociates and transcription begins anew, our observations provide a basis for further experimentation to address both possibilities.

Although it is possible that the role of TFIIIE in transcription is primarily to recruit TFIIH, an analysis of the primary sequence of the TFIIIE subunits has revealed computer-predicted similarities to several interesting structural features suggesting that TFIIIE may serve additional functions. For example, the observation that TFIIIE possesses structural similarities to the bacterial DNA repair protein UvrA (Peterson et al. 1991) is suggestive of a direct involvement of TFIIIE in the process of DNA repair. In addition to UvrA similarity, TFIIIE has been predicted to have a zinc coordination domain, a nucleotide-binding site, and a region similar to the catalytic domain of several protein kinases. The precise mechanism by which these predicted features of TFIIIE may contribute to the processes of transcription and DNA repair is currently under investigation using various strategies employing mutagenesis. The analysis of recombinant TFIIIE subunits carrying mutations targeted to computer-predicted structural features in reconstituted transcription, affinity interaction, and DNA repair assays is expected to facilitate the characterization of discrete domains that may be responsible for governing the activities of TFIIIE in both transcription and transcription-coupled DNA repair.

Materials and methods

Generation of polyclonal antibodies against TFIIIE subunits

Polyclonal antisera against TFIIIE-56 and TFIIIE-34 were raised in rabbits using *Escherichia coli*-expressed recombinant proteins, which were purified as described previously (Peterson et al. 1991). Antigens were prepared using Freund's complete and incomplete adjuvant, and injections were done subcutaneously. Antisera were tested for reactivity and specificity in Western assays.

Protein-protein interaction assays

Coimmunoprecipitation assays were performed as described previously (Yokomori et al. 1993a) using 2 μ l of α -TFIIIE-56 or α -TFIIIE-34 antiserum, where indicated, and 0.1 M KCl/HEMG-binding buffer containing 0.01% NP-40 and 0.5 mM DTT.

RNA Pol II containing a mixture of RNA Pol II α and RNA Pol II β forms was purified as described previously (Lu et al. 1991), and ~250–400 ng was used per assay. Where indicated, 300 and 350 ng of recombinant TFIIIE-56 and TFIIIE-34 were used, respectively, in assays. Coimmunoprecipitations were done in binding buffer described above, and beads were washed extensively and boiled in SDS-PAGE sample buffer prior to loading on 6% gels. Proteins were visualized after transfer to nitrocellulose using a

1:5000 dilution of a monoclonal antibody specific for the large subunit of RNA Pol II obtained from Promega.

TFIIF interaction assays were performed as described for RNA Pol II interaction assays using immunoaffinity resins for TFIIIE-34 and TFIIIE-56, with the following modifications. RAP30 and RAP74 were radiolabeled using the TNT T7 Coupled Reticulocyte Translation system available from Promega. Recombinant TFIIIE subunits were prebound to the appropriate immunoaffinity resin, the beads were washed extensively, and radioactive TFIIF subunits were added as indicated in the legend to Figure 2. Beads were washed extensively after a 2 hr incubation, resuspended in SDS-PAGE sample buffer, and run on a 10% SDS-PAGE gel prior to visualization via autoradiography.

TBP coimmunoprecipitation assays were performed using anti-TFIIIE-56 antiserum, 300 ng of TFIIIE-56, and 150 ng of recombinant human TBP purified from *E. coli* as described previously (Peterson et al. 1990) and visualized by Western analysis using a monoclonal antibody specific for TBP (Lobo et al. 1992), kindly provided by Nouria Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

TBP interaction assays using glutathione-agarose beads were performed as described previously (Goodrich et al. 1993), with the following modifications. [35 S]Methionine labeling of proteins was performed using the TNT T7 Coupled Reticulocyte Translation system available from Promega. Proteins were incubated with the GST and GST-chimera beads for 2 hr at 4°C. The total amount of GST fusion protein was ~1.5 μ g, and the concentrations of KCl used are indicated in the legend to Figure 3. Where radioactive proteins were used, SDS-PAGE gels were dried and exposed to film.

TFIIH interaction assays were performed using beads containing 0.5 μ g of GST-56 fusion protein and 40 μ l of a partially purified TFIIH fraction (Flores et al. 1992) in 0.1 M KCl HEMG binding buffer. The beads were washed extensively and resuspended in SDS-PAGE sample buffer prior to boiling and loading onto a 10% SDS-polyacrylamide gel. The bound material was visualized by Western blot using 1:1000 dilution of antibodies against the p62 subunit of TFIIH.

TFIIH interaction/elution assays were performed using beads containing 0.7 μ g of GST-56 fusion protein and 100 μ l of a partially purified phenyl-Superose TFIIH fraction (Flores et al. 1992). The incubation was performed at 4°C for 1.5 hr in HEMG binding buffer containing 0.1 M KCl and 4 mM MgCl₂. The beads were washed extensively and eluted for 45 min using 100 μ l of binding buffer containing 0.5 M KCl, and subsequently with 100 μ l buffer containing 1.0 M KCl. Eluted material was dialyzed immediately into 0.1 M binding buffer for 3 hr prior to transcription assay analysis. Eluted material was also resolved on a 10% SDS-polyacrylamide gel and visualized by Western blot using 1:1000 dilution of antibodies against the p62 subunit of TFIIH.

Interaction assays using amylose beads available from New England Biolabs were performed under conditions described for glutathione-agarose beads, with the following modification. The MBP-ERCC-3 fusion protein concentration was ~3 μ g per assay and 0.15 M KCl HEMG-binding buffer was used. [35 S]Methionine in vitro-translated TFIIIE subunits were visualized via autoradiography.

All input lanes represent 10% of the amount of the indicated proteins tested in the assays.

In vitro transcription

In vitro transcription assays were performed in a HeLa transcription system reconstituted with recombinant human TBP, TFIIB, TFIIIE, and TFIIF, highly purified RNA Pol II and a partially purified TFIIH fraction. The following recombinant pro-

teins were expressed in *E. coli* and purified as described: TBP (Peterson et al. 1990); TFIIB (Ha et al. 1991); TFIIE-34 and TFIIE-56 (Peterson et al. 1991); and RAP30 and RAP74 (Aso et al. 1992; Finkelstein et al. 1992). TFIIF was purified as described (Flores et al. 1992) and was a kind gift from Lalo Flores. RNA Pol II was purified from HeLa cell nuclear pellets through the DEAE-5PW step as described (Lu et al. 1991). Transcription reactions were performed in a final volume of 25 μ l in buffer containing 20 mM Tris-Cl (pH 7.9), 10% glycerol, 1 mM DTT, 4 mM MgCl₂, 50 mM KCl, and 10 mM ammonium sulfate. Each reaction contained 20 ng of TBP, 20 ng of TFIIB, 40 ng of RAP30, 10 ng of RAP74, 30 ng of TFIIE-34, 40 ng of TFIIE-56, 125 ng of RNA Pol II, and 200 ng of template DNA containing the adenovirus major late promoter (−53 to +10) upstream of the G-less cassette (Sawadogo and Roeder 1985). The reactions did not contain 3'-O-Methyl GTP or RNase T1. Transcription factors were preincubated with DNA for 30 min at 30°C. Nucleotides (2 μ l of a 13 $\times$ mixture) were added to give final concentrations of 500 μ M ATP, 500 μ M CTP, and 25 μ M [α -³²P]UTP (5 μ Ci). Transcription was allowed to proceed for 15 min at 30°C. Reactions were stopped by the addition of 100 μ l of a solution containing 3.1 M ammonium acetate, 10 μ g of carrier yeast RNA, and 15 μ g of proteinase K. The samples were subjected to denaturing 6% PAGE after ethanol precipitation, and the transcripts were subsequently visualized by autoradiography.

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