TAFII-independent activation mediated by human TBP in the presence of the positive cofactor PC4

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TFIID is a multiprotein complex comprised of the TATA-binding protein (TBP) and an array of TBP-associated factors (TAFII8). Whereas TBP is sufficient for basal transcription in conjunction with other general transcription factors and RNA polymerase II, TAFII8 are additionally required for activator-dependent transcription in mammalian cell-free transcription systems. However, recent in vivo studies carried out in yeast suggest that TAFII8 are not globally required for activator function. The discrepancy between in vivo yeast studies and in vitro mammalian cell-free systems remains to be resolved. In this study, we describe a mammalian cell-free transcription system reconstituted with only recombinant proteins and epitope-tagged multiprotein complexes. Transcriptional activation can be recapitulated in this highly purified in vitro transcription system in the absence of TAFII8. This TBP-mediated activation is not induced by human mediator, another transcriptional coactivator complex potentially implicated in activator response. In contrast, general transcription factors TFIH and TFIIA play a significant role in TBP-mediated activation, which can be detected in vitro with Gal4 fusion proteins containing various transcriptional activation domains. Our data, therefore, suggest that TFIH and TFIIA can mediate activator function in the absence of TAFII8.

Keywords: TAFII8/TBP/TFIIA/TFIIH/transcriptional activation

Introduction

In prokaryotes, transcription is initiated by RNA polymerase holoenzyme which contains core RNA polymerase (αββ′) in association with a σ factor that recognizes the core promoter elements. Bacterial activators usually enhance transcription through interactions with different σ factors or components of core RNA polymerase (Geridushek, 1997; Hochschild and Dove, 1998). In eukaryotes, transcription of class II promoters by RNA polymerase II (pol II) generally begins with TFIID binding to the TATA box, which is followed by the assembly of other general transcription factors (GTFs) including TFIIA, TFIIIB, TFIIE, TFIIH and pol II on the promoter region (Conaway and Conaway, 1993; Buratowski, 1994; Zawel and Reinberg, 1995; Orphanides et al., 1996; Roeder, 1996). Formation of the preinitiation complex (PIC) is often facilitated by upstream sequence-specific transcription factors which promote PIC assembly by interacting with essentially any component of the general transcription machinery. Another pathway for PIC assembly is via a preassembled pol II holoenzyme complex which contains pol II in association with a subset of GTFs and other protein factors implicated in chromatin remodeling, DNA repair or mRNA processing (Kim et al., 1994; Koleske and Young, 1994; Ossipow et al., 1995; Chao et al., 1996; Maldonado et al., 1996; Wilson et al., 1996; Yuryev et al., 1996; McCracken et al., 1997; Wu and Chiang, 1998).

TFIID, a multiprotein complex consisting of the TATA-binding protein (TBP) and an array of TBP-associated factors (TAFII8), has been implicated as a major target by various transcriptional activators (Burley and Roeder, 1996; Verrijzer and Tjian, 1996; Tansey and Herr, 1997). Previous in vitro studies indicated that TBP, in conjunction with other GTFs and pol II, supports only basal transcription from core promoter elements (Hoffmann et al., 1990; Peterson et al., 1990; Pugh and Tjian, 1990). Activation of transcription in these cell-free systems relies strictly on the presence of TAFII8 as well as the upstream stimulatory activity (USA) cofactor fraction (Meisterernst et al., 1991; Chiang et al., 1993). Further fractionations of USA led to the identification of several positive cofactors (PCs) including PC1, PC2, PC3 and PC4, and negative cofactors (NCs) (Kaiser and Meisterernst, 1996). The role of TAFII8 in mediating activator function has recently been questioned by in vivo studies carried out in yeast (Apone et al., 1996; Moqtaderi et al., 1996; Walker et al., 1996). Although TAFII8 also seem to be required for activator function in the Drosophila embryo (Sauer et al., 1996), the discrepancy of the role of TAFII8 in activator response between yeast and multicellular organisms remains to be resolved.

To define the role of TBP and TAFII8 in transcriptional activation and to avoid potential complications due to contaminants present in our previous transcription systems (Chiang et al., 1993; Chiang and Roeder, 1995), we first refined our cell-free transcription system by using only recombinant factors (TFIIA, TFIIB, TBP, TFIIE, TFIIH, PC4 and Gal4–VP16) and highly purified epitope-tagged multiprotein complexes (TFIID, TFIIH and pol II). In this highly purified transcription system, TAFII8 indeed contributed to a high level of activation as observed in previous systems. Surprisingly, we were also able to detect TBP-mediated activation by Gal4 fusion proteins with different activation domains in our cell-free transcription system. Since components of human mediator (or SRBs, Suppressors of RNA polymerase B mutations; Kim et al., 1994; Koleske and Young, 1994) such as cdk8 and cyclin C were not detected in our highly purified epitope-tagged
protein complexes, it is unlikely that SRBs are responsible for the observed TBP-mediated activation. In contrast, we found that the level of this TAF₈₀-independent activation mediated by human TBP was affected strongly by the amounts of TFIIH and TFIIIA used in the assays. Our data thus suggest that TFIIH and TFIIA may mediate activator response when TAF₈₀s are not present in the system.

**Results**

**Establishment of a highly purified in vitro transcription system**

Over the last few years, cDNAs encoding various human GTFs and pol II subunits have been cloned (Orphanides et al., 1996; Roeder, 1996; Acker et al., 1997; Khazak et al., 1998). Recombinant TFIIA, TFIIIB, TFIIIE and TFIIIF are fully functional in mediating the transcriptional process. However, the difficulty in obtaining highly purified TFIIID, TFIIH and pol II has hampered studies of eukaryotic transcription. By using epitope-tagging and creating stable cell lines, we have now purified human TFIIH and pol II from clonal cell lines that conditionally express the FLAG-tagged p62 subunit of human TFIIH and the FLAG-tagged RPB9 subunit of human pol II, respectively (see Materials and methods). Both epitope-tagged multiprotein complexes (TFIIH and pol II) and the FLAG-tagged human TFIID (Chiang et al., 1993) contain previously defined polypeptides (Figure 1A), but are devoid of human SRB7, cyclin C and cdk8 as judged by Western blotting at a sensitivity of 0.8 ng for cdk8 and 0.4 ng for cyclin C (Figure 1B and data not shown). This indicates that the mediator, a multiprotein complex defined initially in the yeast system (Kim et al., 1994; Koleske and Young, 1994) and which also possibly functions as a general coactivator, is not present in our transcription system. Furthermore, components of TFIID are not detected in epitope-tagged pol II and TFIIH complexes by Western blotting with various anti-TAFs antibodies at a sensitivity of 1 ng for TBP and 1 ng for TAF₈₀ (Figure 1B), indicating that TFIID is the only source of TAF₈₀s in our highly purified in vitro transcription system. The presence of pol II and TFIIH in this experiment is demonstrated by anti-RPB2 and anti-MAT1 antibodies.

Fig. 1. Protein factors used in the reconstituted transcription assays. (A) Silver staining of purified FLAG-tagged protein complexes. Purification of FLAG-tagged TFIIID (left lane), FLAG-tagged TFIIH (central lane) and FLAG-tagged pol II (right lane) was performed as described in Materials and methods. The assignment of various TFIID subunits was based on Chiang et al. (1993) and was also confirmed by Western blotting with anti-TBP and anti-TAF₈₀ antibodies. The positions of individual TFIIH and pol II subunits were all confirmed by Western blotting except those of RPB3, RPB5, RPB10ε/β and RPB11, which were assigned based on the published molecular weights (Acker et al., 1997; Khazak et al., 1998). (B) The mediator components cdk8 and cyclin C are not present in purified FLAG-tagged protein complexes. Western blotting was performed as described in Materials and methods with the primary antibodies indicated on the right. The amounts (ng) of recombinant cdk8, cyclin C, FLAG-tagged TBP and FLAG-tagged TAF₈₀ used as protein standards for quantitative Western blotting were depicted on the left. (C) Coomassie Blue staining of purified recombinant human general transcription factors and PC4. Transcription factors TFIIA (A: p55, p35, p19 and p12), TFIIIB (B), TBP (T), TFIIIEα (Eα), TFIIIEβ (Eβ), TFIIIF (F) subunits RAP30 (30) and RAP74 (74) and PC4 were purified as described in Materials and methods. Prestained protein size markers (in kDa) are indicated on the left.
respectively (Figure 1B). The other human general transcription factors (TFIIA, TFIB, TFIE, TFIF) and a coactivator PC4 (Ge and Roeder, 1994a; Kretzschmar et al., 1994) were expressed and purified from bacteria (Figure 1C). Gal4–VP16 (Chasman et al., 1989) was used as an activator. The transcription template pG5HMC2AT contains five Gal4-binding sites preceding the HIV-1 TATA box and the adenovirus major late promoter (MLP) initiator element in front of a G-less cassette of ~380 nucleotides, whereas pMLA53, which lacks the activator-binding sites, has a shorter G-less cassette (~280 nucleotides) driven only by the MLP TATA and initiator elements. The entire set of transcription components was then reconstituted in vitro for the mechanistic studies of basal and activator-dependent transcription.

To determine whether all GTFs are required for basal transcription in our highly purified transcription system, we first combined all GTFs and pol II with supercoiled DNA templates (pG5HMC2AT and pMLA53) in a single reaction tube. Individual factors were then omitted from the complete reaction. As shown in Figure 2A, TFIIB, TFIF, pol II and a TATA-binding activity (either TBP or TFIIID) were essential for transcription (Figure 2A, lanes 3, 4, 6 and 8), whereas TFIIE and TFIIH, although they might not be necessary for transcription from supercoiled DNA templates (Goodrich and Tjian, 1994; Parvin et al., 1994; Timmers, 1994; Figure 2A, lanes 5 and 7), were required for transcription from linearized DNA molecules (Figure 2B). Interestingly, transcription from pG5HMC2AT and pMLA53 seems to require different amounts of TFIIE and TFIIH (Figure 2A, lanes 5 and 7). Obviously, TFIIA and TAF15s were not needed for basal transcription from either DNA template (Figure 2A, compare the top panel with the bottom panel, and lane 1 with lane 2), consistent with previously published results (Orphanides et al., 1996; Roeder, 1996).

We also examined the GTF requirement for TFIIID-mediated activator-dependent transcription in this highly purified in vitro transcription system. All GTFs except TFIIA were necessary, in conjunction with Gal4–VP16 and PC4, for activated transcription (Figure 2C, lanes 1–8). Gal4–VP16, in the absence of PC4, could not activate transcription (Figure 2C, compare lane 9 with lane 11), consistent with the concept that at least a general coactivator in addition to TAF15s is required for activator-dependent transcription (Meisterernst et al., 1991; Chiang et al., 1993; Ge and Roeder, 1994a; Kretzschmar et al., 1994). This experiment also demonstrates that there was only negligible, if any, cross-contamination of factors in our transcription components. Surprisingly, leaving out TFIIA had no effect on activated transcription in this highly purified in vitro transcription system (Figure 2C, compare lane 1 with lane 2). This result was distinct from previous observations which indicated that TFIIA was usually required for activator-dependent transcription, although it may not be necessary for basal transcription (Ma et al., 1993; Ozer et al., 1994; Sun et al., 1994; Yokomori et al., 1994; DeJong et al., 1995; Kobayashi et al., 1995; Chi and Carey, 1996; Shykind et al., 1997 and references therein). TFIIA was apparently not present in our nearly homogeneous preparation of TFIIH and pol II (which were purified respectively under 0.5 and 1.0 M urea washing conditions, see Materials and methods), since no TFIIA could be detected in any of these protein complexes by Western blotting (data not shown). However, to rule out any potential TFIIA contamination that may contribute functionally to the transcriptional activity and could not be detected by Western blotting, we also carried out in vitro transcription reactions using epitope-tagged TFIIID that was purified under high salt (0.5 or 0.85 M KCl) washing conditions, as TFIIA has been shown to dissociate from TFIIID in 0.5 M salt (Yokomori et al., 1993). Again, TFIIA was not required for activated transcription under these experimental conditions (data not shown).

**TFIIA becomes necessary for activated transcription when partially purified TFII/E/F/H or USA fractions are used in the transcription assay**

In our previous transcription system reconstituted with recombinant TFIIIB, epitope-tagged TFIIID, but partially
purified TFII/E/F/H and USA fractions, TFIIA was clearly required for activator-dependent transcription (Chiang et al., 1993; Ma et al., 1993; Ozer et al., 1994; Sun et al., 1994; Yokomori et al., 1994; Chiang and Roeder, 1995; DeJong et al., 1995; Kobayashi et al., 1995; Shykind et al., 1995, 1997; Chi and Carey, 1996 and references therein). Presumably TFIIA was only needed to antagonize the repressive effect from some negative factors present in the transcription systems containing relatively crude fractions. To test this hypothesis, we performed an in vitro transcription assay using combinations of recombinant proteins and partially purified column fractions. As shown in Figure 3, Gal4–VP16-mediated activation in our purified transcription system reconstituted with recombinant (r) TFIIA, rTFIIE, rTFIIF, rPC4, FLAG-tagged TFID, FLAG-tagged TFIIH and FLAG-tagged pol II was not further enhanced by the addition of either recombinant or native (i.e. column-purified) TFIIA (lanes 1–4). When USA was used in place of PC4, the activated transcription, performed in the absence of TFIIA, was reduced (Figure 3, compare lane 2 with lane 10), consistent with the fact that USA also contains negative cofactors in addition to positive cofactors. Interestingly, under the reduced level of activator-dependent transcription, native TFIIA promoted a high level of activation (Figure 3, compare lane 9 with lane 12). Recombinant TFIIA, although functional in enhancing TBP-mediated activation (see below), did not show any effect on this TFIID-dependent transcription assay. Similar results were obtained when a partially purified TFII/E/F/H fraction was substituted for rTFIIIE, rTFIIF and FLAG-tagged TFIIH (Figure 3, lanes 5–8 and 13–16). Although the overall transcription activity provided by the TFII/E/F/H fraction was lower, native TFIIA was clearly required for an optimal level of activation, irrespective of whether PC4 or USA was used in the assay. These results indicated that native TFIIA might contain additional cofactor activity that could further enhance Gal4–VP16 activation when USA or the TFII/E/F/H fraction was used. This cofactor activity, however, was not present in our highly purified transcription system. Our data are in agreement with the observation that an activity (cofactor A) present in the phosphocellulose 0.1 M KCl fraction was required to achieve an optimal level of activation when native or recombinant TFIIA was used in conjunction with USA (Sun et al., 1994; Maldonado et al., 1996). Alternatively, post-translational modification may play a role in modulating TFIIA function, which can also account for the variation in native and recombinant TFIIA activity.

**TFIIH has a significant effect on TFIIID- and TBP-mediated activation**

After defining the requirement for GTFs in our transcription system, we began to address the role of TBP and TAF15s in activator-dependent transcription. In a previous study using a preassembled pol II holoenzyme complex in comparison with individually reconstituted GTFs (Wu and Chiang, 1998), we found that the amount of TFIIH used in the assay had a significant effect on the level of activated transcription. Therefore, we first examined the effect of TFIIH concentrations on TFIIID-mediated transcription. At low concentrations of TFIIH, Gal4–VP16 alone could not activate transcription (Figure 4A, compare lane 1 with lane 2, and lane 5 with lane 6). As expected (Malik et al., 1998; Wu and Chiang, 1998), PC4, in the absence of Gal4–VP16, repressed basal transcription from both DNA templates (Figure 4A, compare lane 1 with lane 3, and lane 5 with lane 7). The activator was then able to reverse PC4 repression and stimulate activation above the original basal level (Figure 4A, compare lane 1 with lane 4, and lane 5 with lane 8). When compared with PC4-suppressed basal transcription, Gal4–VP16 activated overall transcription up to 107-fold (Figure 4A, compare lane 3 with lane 4, and lane 7 with lane 8). A further increase of TFIIH had little, if any, effect on the basal transcription from pMLΔ53, although it enhanced the basal transcription from pG5HMC2AT (Figure 4A, lanes 1, 5, 9, 13 and 17), consistent with the result which indicated a differential requirement of TFIIH for transcription from pG5HMC2AT and pMLΔ53 (Figure 2A). Interestingly, PC4 repression of basal transcription was clearly overcome by increasing amounts of TFIIH (Figure 4A, compare lane 9 with lane 11, lane 13 with lane 15, and lane 17 with lane 19). Under the derepressed state, TFIIH further enhanced Gal4–VP16 activation up to ~30-fold (Figure 4A, compare lane 17 with lane 20). We then investigated the effect of TFIIH on TBP-mediated transcription. Surprisingly, in our highly purified in vitro transcription system, we were able to detect for the first time that TBP, in the absence of TAF15s, could
mediate Gal4–VP16 activation (Figure 4B). At low concentrations of TFIIH, Gal4–VP16 alone could not activate transcription and PC4 suppressed basal transcription, as observed in TFIID-mediated transcription (compare Figure 4A and B, lanes 1–3 with lanes 5–7). However, TBP-mediated activation was clearly observed when both Gal4–VP16 and PC4 were present (Figure 4B, compare lane 3 with lane 4, and lane 7 with lane 8). This stimulation process is activator-dependent and occurs only on the DNA template containing the activator-binding sites. A further increase of TFIIH, as in the case of TFIID-mediated transcription, enhanced the basal transcription from pG5HMC2AT, but only marginally from pMLΔ53, indicating that TFIIH is limiting in this transcription system. This was also reflected by the observation that Gal4–VP16 slightly inhibited basal transcription from both DNA templates (Figure 4B, compare lane 1 with lane 2, lane 5 with lane 6, lane 9 with lane 10, lane 13 with lane 14, and lane 17 with lane 18), as interactions between Gal4–VP16 and TFIIH might squelch (or titrate out) TFIIH further away from the promoter region. Again, PC4 repression of basal transcription was overcome gradually by increasing amounts of TFIIH, although the alleviation seemed less efficient in the absence of TAFIIs (compare Figure 4A with B, lane 9 with lane 11, lane 13 with lane 15, and lane 17 with 19). This observation was consistent with the result that high concentrations of TFIIH, but not TBP, could also overcome PC4 repression (Wu and Chiang, 1998). Interestingly, TBP-mediated activation, when normalized with the original basal level, was more pronounced at high concentrations of TFIIH (Figure 4B, compare lane 1 with lane 4, lane 5 with lane 8, lane 9 with lane 12, lane 13 with lane 16, and lane 17 with lane 20).

The effect of TFIIH on TBP-mediated activation was also observed on a different DNA template (pG5MLT) containing five Gal4-binding sites linked to the natural MLP TATA and initiator elements (Figure 4C). In this experiment, both DNA templates (pG5MLT and pMLΔ53) contain the same core promoter region but one has additional activator-binding sites. Consistent with the previous results (Figure 4A and B), increasing amounts of TFIIH not only enhanced TBP-mediated activation with
TBP-mediated activation

Fig. 5. The effect of TFIIH on transcriptional activation mediated by various Gal4 fusion proteins in the presence or absence of TAFIIs. In vitro transcription was performed with either 1 ng of FLAG-tagged TBP (top panel) or an equivalent TBP content of FLAG-tagged TFIID (bottom panel), in the presence (+) or absence (−) of PC4 and various Gal4 fusions as indicated. Recombinant Gal4 fusion proteins used are: Gal4–VP16 (VP16), FLAG-tagged Gal4–Pro (Pro), FLAG-tagged Gal4–Gln (Gln), FLAG-tagged Gal4 (1–147) and FLAG-tagged Gal4 (1–94). The number above each lane is the fold activation (for the pG5MLT template) as defined in the legend to Figure 4C.

To see whether TFIIH also has a significant effect on TBP- and TFIID-mediated activation by other transcriptional activators, we tested the ability of various Gal4 fusions in activating transcription in this highly purified in vitro transcription system. Both Gal4–Pro and Gal4–Gln, which contain proline-rich and glutamine-rich activation domains linked respectively to the Gal4 DNA-binding domain, were able to activate transcription mediated by TBP and TFIID at different TFIIH concentrations, although their activation levels were not as efficient as that achieved by the potent activator Gal4–VP16 (Figure 5, top and bottom panels, compare lanes 3–5 with lane 2, and lanes 10–12 with lane 9). In contrast, Gal4 (1–94) which was the DNA-binding domain used in Gal4–Pro and Gal4–Gln had only minor if any effect on transcription (Figure 5, top and bottom panels, compare lane 2 with lane 7, and lane 9 with lane 14). Interestingly, Gal4 (1–147) which was employed to make Gal4–VP16 was capable of activating transcription to a level similar to that seen with Gal4–Pro and Gal4–Gln (Figure 5, top and bottom panels, lanes 2–6 and 9–13). This was consistent with the observation that an activation domain was present between amino acids 75 and 147 and was fully functional in vitro (Lin et al., 1988; Ge and Roeder, 1994a; Wu and Chiang, 1998). This might explain why Gal4–VP16 was such a potent transcriptional activator, presumably two activation domains were used synergistically to activate transcription.

As observed previously (Malik et al., 1998; Wu and Chiang, 1998; Figure 4), both TAFIIs and increasing amounts of TFIIH could alleviate PC4 repression of the basal transcription in the absence of an activator (Figure 5, compare lanes 2 and 9 in the top panel with lanes 2 and 9 in the bottom panel, and lane 2 with lane 9 in the same panel).

TFIIA also enhanced TBP-mediated activation by Gal4–VP16

Since TBP-mediated activation could be enhanced significantly by increasing concentrations of TFIIH, we wondered whether other components of the general transcription machinery might also affect this TAFII-dependent activation process. To explore this possibility, we tested whether TBP-mediated activation could be enhanced further by adding increasing amounts of individual GTFs to a transcription reaction where Gal4–VP16 activated
transcription only marginally (Figure 6A, lanes 1–4). As expected, increasing amounts of TFIIH dramatically stimulated TBP-mediated activation by Gal4–VP16 (Figure 6A, lanes 15 and 16). With the exception of TFIIA, other GTFs had little, if any, effect on TBP-mediated activation (Figure 6A, lanes 5–18). To see whether TFIIA-stimulated transcription was due to an enhancement of the basal transcription or to true activation, we carried out a more detailed transcription analysis. As shown in Figure 6B, increasing amounts of TFIIA further enhanced activator-dependent transcription but showed little effect on basal transcription, indicating that TFIIA could indeed stimulate TBP-mediated activation.

Since TFIIA had some effect on TBP-mediated activation which could also be affected significantly by the amount of TFIIH used in the assays, we wondered whether TFIIA could potentiate TFIIID-mediated activation by Gal4–VP16 under different TFIIH concentrations. As shown in Figure 7, TFIIA had no effect on TFIIID-mediated activation at either low or high concentrations of TFIIH tested (Figure 7, lanes 1–8), consistent with the previous assays indicating that TFIIA was not required for TFIIID-mediated activation by Gal4–VP16 (Figure 2C, lanes 1 and 2; Figure 3, lanes 2 and 3). Because the requirement for TFIIA could also be influenced by the concentration of TFIIID used in the assay (Lieberman et al., 1997 and references therein), we tested if TFIIA was necessary in our reconstituted transcription system when TFIIID became limiting. As shown on the right side of the panel (Figure 7, lanes 9–16), TFIIA still had no effect on Gal4–VP16 activation under conditions where TFIIID was the limiting factor as reflected by the reduction of the basal transcription from both DNA templates (Figure 7, compare lane 1 with lane 9, lane 3 with lane 11, lane 5 with lane 13, and lane 7 with lane 15). Moreover, enhancement of TFIIID-mediated Gal4–VP16 activation by increasing amounts of TFIIH was more evident at limiting TFIIID concentrations (Figure 7, compare lane 2 with lane 6, and lane 10 with lane 14).

Discussion

Using a highly purified mammalian cell-free transcription system reconstituted with only recombinant proteins and epitope-tagged protein complexes, we were able to demonstrate that human TBP could indeed mediate activator function, in the absence of TAFIIs. This is the first documentation where it has been shown unambiguously that TAFII-independent activation can occur in a mammalian cell-free transcription system reconstituted with individually purified general transcription factors, consistent with the observations in the yeast studies (Koleske and Young, 1994; Apone et al., 1996; Moqtaderi et al., 1996; Walker et al., 1996). Although TBP might appear to enhance transcription in some of the transcription systems reported previously (Workman et al., 1990; Chiang et al., 1993; Ge and Roeder, 1994a), the presence of TAFIIs could not be excluded owing to the use of cruder protein
fractions in these experiments. In our reconstituted transcription system, TFIID was the only source of TAFIIs which was not found in the other transcription components including FLAG-tagged TFIIH and FLAG-tagged pol II as shown by Western blotting with anti-TBP and anti-TAFII antibodies (Figure 1B). The leave-out transcription assays further demonstrated that without TFIID or TBP no transcription could be detected (Figure 2A and C). These results indicate that, even if there were minor TAFIIs present in other transcription components which were undetectable by Western blotting, these contaminants could not functionally account for the TFIID activity. Our transcription system also rules out the contribution of the mediator in TBP-mediated activation, since components of the human mediator such as SRB7, cyclin C and cdk8 could not be detected by Western blotting in any of our transcription components (Figure 1B and data not shown).

Moreover, it was unlikely that mediator components would associate with FLAG-tagged TFIIH and FLAG-tagged pol II which were isolated, respectively, after 0.5 and 1.0 M urea-containing high salt washing conditions. This individually reconstituted transcription system also allowed us to carefully titrate each transcription component used in the assays. We found that TFIIH and TFIIA could enhance TBP-mediated activation by Gal4–VP16. Therefore, this highly purified reconstituted transcription system provides us with a unique opportunity to dissect the molecular basis of TBP-mediated activation.

In vivo relevance of TBP-mediated activation

In Drosophila and human cells, TFIID is isolated as a tightly associated multiprotein complex which can only be disrupted by using strong chaotropic agents, such as 3 M urea (Dynlacht et al., 1991; Tanese et al., 1991). In contrast, yeast TFIID seems to be a loosely associated complex and can only be isolated under mild chromatographic conditions (Reese et al., 1994; Poon et al., 1995). Nevertheless, it is possible that TBP is generated in vivo when some yeast TAFIIs essential for the integrity of TFIID are not present in the cell (Walker et al., 1996) or in vitro when HeLa nuclear extracts are heat-treated at 47°C for 15 min (Nakajima et al., 1988). The composition of some TAFIIs important for TFIID assembly may vary at certain developmental stages or at particular cellular differentiation pathways, creating conditions in which the TFIID complex would dissociate. The free form of TBP would then be able to mediate activator response. Several observations may correlate with this possibility. First, some human TAFIIs are selectively depleted in specific cell types (May et al., 1996; S.-Y. Wu and C.-M. Chiang, unpublished data). Secondly, the functions of many TAFIIs are implicated in cell-cycle progression (Wang and Tjian, 1994; Walker et al., 1996, 1997; Apone et al., 1996; Suzuki-Yagawa et al., 1997). Thirdly, some TAFIIs become limiting or degraded in the stationary phase when cells are no longer dividing (Walker et al., 1997). Fourthly, the free form of TBP is occasionally found during chromatographic fractionations of HeLa nuclear extracts (C.-M. Chiang, unpublished data), hence free TBP may be present in some stationary phase cells before the preparation of nuclear extracts. It is therefore conceivable that in some terminally differentiated cells or specific cell types, TBP is freed from TFIID or other TBP-containing complexes such as SL1, B-TFIID, TFIIIB and SNAPc because of the loss of some TAFs essential for complex assembly, thereby creating an environment for TBP-mediated activation. This possibility remains to be investigated in the future.

GTFs required for basal and activated transcription

The requirement of GTFs for basal and activated transcription varies depending on the promoters and structures of the DNA templates. In general, transcription from supercoiled DNA templates does not require TFIIH (Goodrich and Tjian, 1994; Parvin et al., 1994; Timmers, 1994). However, in our transcription system, leaving out TFIIE and TFIIH seems to have a dramatic effect on transcription from the HIV promoter (pG4HMC2AT), but has less effect on transcription from the adenovirus major late promoter (pMLA53) (Figure 2A, top and bottom panels, compare lane 1 with lane 5 and lane 7). Whether a differential requirement of TFIIIE and TFIIH for pG4HMC2AT and pMLA53 is caused by variation on the DNA templates remains to be defined. Given the observations that HIV Tat interacts functionally with TFIIH and that the stalled HIV 5′ transcripts can be efficiently extended via Tat enhancement of the phosphorylation of the C-terminal domain of RNA polymerase II by TFIIH (Parada and Roeder, 1996; Cujec et al., 1997; Garcia-Martinez et al., 1997; Jones, 1997 and references therein), our data imply that recruitment of TFIIH may be an important step in HIV transcription.
The observation that TFIIIE can stimulate promoter activity from both the HIV and adenovirus major late promoters in our transcription system is also consistent with a previous report demonstrating that transcription from the adenovirus major late promoter is more dependent on TFIIIE than transcription from adenovirus E4 and mouse mammary tumor virus (MMTV) promoters (Holstege et al., 1995). Furthermore, transcription becomes increasingly dependent on TFIIIE when helical opening of supercoiled DNA templates is suppressed by higher ionic strengths (Holstege et al., 1995). These results indicate that the requirement of TFIIIE is also determined by the promoter structure and variables that affect the helical stability of DNA topology such as ionic strength and the MgCl₂ concentrations, in addition to its role in recruiting TFIIH to the promoter region (Holstege et al., 1995). In a similar way, whereas TFIIF is essential for most promoter functions, it is not required for transcription from the IgH (immunoglobulin heavy chain) promoter (Parvin et al., 1994).

In addition to the promoters and structures of the DNA templates, the purity of the transcription system also affects the requirement for GTFs. In our highly purified transcription system, TFIIA is not required for either basal or activated transcription (Figure 2). By varying the purity of transcription components, we further demonstrate that TFIIA becomes necessary when partially purified protein fractions such as TFIIIE/H and USA are used in the transcription assays (Figure 3), indicating that TFIIA is needed to antagonize the repressive effect from some negative factors that may be present in transcription systems containing cruder protein fractions. Interestingly, the level of transcriptional stimulation by native TFIIA in conjunction with USA is apparently higher than the level achieved with our highly purified transcription components (Figure 3). This suggests that other protein factors present in partially purified TFIIA and/or USA fractions may work concurrently with TFIIA in enhancing activation in cruder transcription systems, in agreement with the finding that a ‘cofactor A’ is required to stimulate TFIIA function in a transcription system containing the USA fraction (Sun et al., 1994; Maldonado et al., 1996). However, the requirement for TFIIA also depends on the promoter structure and the assay conditions, as it has been shown that conditions which limit TFIID binding to the TATA element or compromise the ability of TFIIA to bind TBP require activator stimulation of the TFIID–TFIIA complex in a cruder transcription system (Lieberman et al., 1997). Therefore, the requirement for individual GTFs should be carefully defined in different transcription systems.

**Repression and antirepression**

*In vivo*, DNA is packaged into nucleosomes which are further condensed via interactions with histone H1 and non-histone chromosomal proteins; thus, the promoter activity of condensed DNA is usually suppressed. To activate transcription, the repressing molecules have to be cleared from the promoter region to allow access and assembly of the transcriptional complex. Some transcriptional activators (Sheridan et al., 1995; Tsukiyama and Wu, 1995; Wong et al., 1995; Armstrong and Emerson, 1996; Brown et al., 1996; Pazin et al., 1996) and coactivators (Ding et al., 1994; Paranjape et al., 1995) help recruit chromatin remodeling factors and/or alleviate nucleosome repression, thereby enhancing transcriptional initiation on chromatin templates (Kingston et al., 1996; Wade et al., 1997; Kadonaga, 1998). Another class of activators (Kraus et al., 1994; Kashanchi et al., 1996) and coactivators (Kaiser and Meisterernst, 1996) work by competing with negative factors for binding to TBP or TFIID. Conceptually, antirepression is an essential process for activator function *in vivo*. When transcription activity is reconstituted *in vitro* using naked DNA templates, stimulation of transcription by activators is usually defined as fold enhancement relative to the core promoter activity measured in the absence of an activator and a coactivator (i.e. true activation). However, the core promoter activity is often suppressed by negative cofactors such as NC1, NC2, DR1, DBF4, HMG1 and topoisomerase I present in cruder protein fractions (Meisterernst and Roeder, 1991; Meisterernst et al., 1991; Inostroza et al., 1992; Kretzschmar et al., 1993; Merino et al., 1993; Ge and Roeder, 1994b; Shykind et al., 1997). In our highly purified transcription system, PC4 suppresses basal transcription in the absence of an activator (Figure 4). This is not surprising given the fact that PC4, being a non-specific DNA-binding protein, inhibits the assembly of a functional preinitiation complex (Wu and Chiang, 1998). However, in the presence of an activator, PC4 acts as a co-activator (Ge and Roeder, 1994a; Kretzschmar et al., 1994). The dual functions of PC4 in the transcriptional process are modulated by TFIIH, since activator-dependent transcription which includes both antirepression and true activation is affected strongly by TFIIH concentrations (Figure 4). Whether PC4 creates conditions which render the transcription reaction dependent on TFIIH’s promoter opening activity in a way similar to linearizing the DNA template remains to be analyzed. The observation that PC4 repression can be alleviated by increasing amounts of TFIID, TFIIH and a preassembled pol II holoenzyme (Malik et al., 1998; Wu and Chiang, 1998; S.-Y.Wu and C.-M.Chiang, unpublished data) suggests that PC4 interactions with components of the general transcription machinery also play a critical role in regulating the promoter activity. This may explain why PC4 repression of basal transcription, in the absence of an activator, was not observed in previous studies (Ge and Roeder, 1994a; Kretzschmar et al., 1994; Kaiser et al., 1995), presumably high amounts of TFIID and TFIIH were present in cruder transcription systems which mask the repressive effect of PC4.

**Role of TFIIH and TFIIA in TBP-mediated activation**

The finding that TFIIH and TFIIA can influence the level of TBP-mediated activation by Gal4–VP16 (Figures 4 and 6) suggests that TFIIH and TFIIA can mediate activator function, independently of TAFIIs. Interaction studies have implicated that TFIIA (Ozer et al., 1994; Kobayashi et al., 1995; Clemons et al., 1996) and TFIIH (Xiao et al., 1994; Léveillard et al., 1996) may be the functional targets for transcriptional activators. Moreover, a recent experiment performed in yeast has demonstrated that the temperature-sensitive phenotype of a yeast TFAP145 mutant can be functionally rescued by overexpression of TFIIA subunits (Kokubo et al., 1998), indicating that TFIIA may substitute for TFAP145 in supporting activator function under some circumstances. This interpretation is consistent with our
finding that TFIIA is able to potentiate TBP-mediated activation in the absence of TAF155. It thus will be of great interest to see if the conditional phenotypes of some yeast TAF155 mutants can also be functionally rescued by overexpression of TFIIH subunits. If so, this will further substantiate the concept that TFIIH can support TBP-mediated activation in vivo.

In our reconstituted system, TFIIH plays multiple roles in the transcriptional process. First, TFIIH is a defined general transcription factor differentially required for basal transcription from various promoters. Secondly, TFIIH can alleviate PC4 repression of the basal transcription when an activator is not present. Thirdly, TFIIH can enhance TBP-mediated activation by various Gal4 fusions with different activation domains. We do not know whether the cdk-activating kinase components (cdk7, cyclin H and MAT1) of TFIIH are responsible for all the activity observed in vitro, or whether different subunits of TFIIH are selectively required for each process. The isolation of multiple forms of TFIIH varying in subunit compositions (Drapkin et al., 1996; Reardon et al., 1996; Garcia-Martinez et al., 1997; Rossignol et al., 1997) further indicates that TFIIH may be involved in fine-tuning various transcription steps as well as other cellular activity. Likewise, a subform of TFIIH consisting of only the β and γ subunits has also been isolated (Ma et al., 1996). Although this TFIIA variant can overcome poissonserase I-mediated repression of basal transcription, it is not able to support Gal4–VP16 activation, indicating that coactivator and antirepression of TFIIA functions can be clearly separated (Ma et al., 1996). It will be interesting to see how these various forms of TFIIA and TFIIH function in TBP-mediated activation.

In vivo, the assembly of a functional preinitiation complex is usually a rate-limiting step and is often facilitated by transcriptional activators which, in most cases, can increase the level of initiation by enhancing the recruitment of TFIIID and/or other components of the basal transcription machinery to the promoter region (Struhl, 1996; Ptashne and Gann, 1997). Activators can interact with multiple targets to affect various steps of preinitiation complex assembly to maximize the transcriptional response (Choy and Green, 1993). Therefore, transcriptional synergy is often observed with multiple activator-binding sites for an activator (Wang et al., 1992; Carey, 1998) or via combinatorial regulation of an enhancerome complex (Kim and Maniatis, 1997; Merika et al., 1998). The observation that TAF155s are not globally required for activator function in yeast is now supported by our in vitro analysis indicating that TBP, in conjunction with TFIIH and/or TFIIA, is able to mediate transcriptional activation. That TBP alone could not activate transcription in the absence of TAF155s in previous cell-free transcription systems is probably due to the repressing functions of negative cofactors (e.g. HMG1, NC1, NC2, etc.) and the limited availability of TFIIH and TFIIA in the cruder assay systems. Although TBP-mediated activation can indeed occur, the presence of TAF155s nevertheless provide more contact surfaces for various activators and coactivators, thereby enhancing transcriptional synergy. Our in vitro transcription system therefore provides us with a unique opportunity to explore the mechanism of TBP-mediated activation which can be potentially regulated by a variety of gene-specific and general cofactors.

Materials and methods

Protein purification

FLAG-tagged TFIIH was purified from a clonal HeLa cell line, F62/H-8, that conditionally expresses the FLAG-tagged p62 subunit of human TFIIH (Wu and Chiang, 1996) following P11 chromatography and immunoaffinity purification. The F62/H-8 cell line was maintained in suspension culture with Joklik medium containing 7.5% calf serum in the presence of tetracycline (1 μg/ml) and selected with G418 (0.6 mg/ml total weight) for 3 days before expansion for the preparation of nuclear extracts and S100. To induce protein expression, cells were pelleted and washed 4 times with 1× PBS in 250-ml conical centrifuge tubes to remove tetracycline. Cells were then resuspended in fresh Joklik medium plus 7.5% calf serum. Nuclear extracts and S100 were prepared from F62/H-8 cells, 4 days after removing tetracycline, as described previously (Dignam et al., 1983). Fractionation of nuclear extracts was performed by loading 100 ml of nuclear extracts onto a column containing ~100 ml of the phosphocellulose P11 resin (Whatman); the column was then eluted sequentially with 0.1, 0.3, 0.5 and 0.85 M KCl-containing buffer (Chiang et al., 1993). The 0.5 M KCl fraction was concentrated by 38% ammonium sulfate precipitation and resuspended in 20 ml of BC100 buffer (Chiang et al., 1993). After dialysis against BC100, 4 ml of the protein sample was incubated with 0.3 ml of M2-agarose (Kodak/IBI) at 4°C for 6–12 h. The immobilized proteins were then washed sequentially with 10 ml of BC300 plus 0.5 M urea and 0.1% Nonidet-P-40 (NP-40); BC100 (5 times for each wash), and finally eluted with 0.3 ml of BC100 containing 0.2 mg/ml FLAG peptide and 0.01% NP-40 (Chiang et al., 1993). Elutions were repeated for a total of 3 times. Purified proteins were then aliquoted and stored at ~80°C after snap freezing in liquid nitrogen.

FLAG-tagged pol II was purified from a clonal HeLa cell line, hRPB9-3, that conditionally expresses the FLAG-tagged RPB9 subunit of human pol II (Wu and Chiang, 1998). Briefly, 10 ml of S100, prepared from hRPB9-3 cells, were incubated with 0.4 ml of M2-agarose at 4°C for 6–12 h. The immobilized proteins were then washed sequentially with BC850/0.1 M urea, BC850, BC100 (5 times for each wash), and finally eluted with 0.4 ml of BC100 containing 0.2 mg/ml FLAG peptide. The rest of protocols were the same as described for the purification of FLAG-tagged TFIIH.

Recombinant PC4 was purified from bacteria harboring pET11a/PC4 as described (Ge et al., 1996). Purification of recombinant FLAG-tagged basal transcription factors including TFIIA (p55, p35, p19 and p12), TFIIIB, TBP, TFIIEx and TFIIEβ, and histidine-tagged TFIIH subunits (RAP50 and RAP74) was performed as described previously (Chiang and Roeder, 1993; Wu and Chiang, 1998). TFIIA and TFIIIB were then individually purified from FLAG-tagged TFIIH by 38% ammonium sulfate precipitation and resuspended in 20 ml of BC100 buffer (Chiang et al., 1998). Purification of FLAG-tagged TFIIH (Chiang et al., 1993), Gal4–VP16 (Chasman et al., 1989) and FLAG-tagged Gal4 fusion proteins (Chiang and Roeder, 1995) was performed as described. Purification of native TFIIA, TFIIEx/F/I and USA was also performed as described (Chiang et al., 1993).

Western blotting

The quantities of proteins loaded on 12% SDS-polyacrylamide gels were: 25 and 150 ng of FLAG-tagged pol II, 7.5 and 45 ng of FLAG-tagged TFIIH, and 1 and 6 ng of TBP equivalent of FLAG-tagged TFIIA. For experiments performed with anti-cdk8 and anti-cyclin C antibodies, however, 1 and 6 ng of TBP equivalent of FLAG-tagged TFIIA was loaded instead. Western blotting was performed as described. Purification of recombinant FLAG-tagged TBP, FLAG-tagged human TAF155 (Chiang and Roeder, 1995), and the cdk8/cyclin C complex (obtained from E.Lees) were also loaded on the gel as protein standards for quantitative Western analysis.

In vitro transcription

Unless specified in individual experiments, in vitro transcription was typically carried out in a 25 μl reaction mixture containing 50 ng of pG3HMC2A, 20 ng of pMLA53, 42 ng of renatured TFIIA (35 ng of p55 and 7 ng of p12), 10 ng of TFIIIB, 1 ng of TBP or 4 μl of FLAG-tagged TFIIH (which contains ~1 ng of TBP as judged by Western
 blotting), 20 ng each of TFIIE or TFIEβ, 28 ng of renatured TFIF (20 ng of RAP74 and 8 ng of RAP30), 2 µl of FLAG-tagged TFIIH (~7.5 ng/µl), and 2 µl of FLAG-tagged pol II (~12.5 ng/µl) using the conditions described previously (Meisterernst et al., 1991). The DNA templates used for experiments described in Figures 4C and 5 are 35 ng of pG5MLT and 35 ng of pMA53. For activator-dependent transcription, 100 ng of PC4 and 50 ng of Gal4–VP16 were also included as specified. The amount of FLAG-tagged Gal4 fusions used was: 3 ng of Gal4 (1–94), 6 ng of Gal4-Pro, 16 ng of Gal4–Gln, and 30 ng of Gal4 (1–111). For the experiments described in Figure 3, 1 µl of each native TFIIA (0.5 mg/ml), TFIE/F/FH (0.3 mg/ml) and USA (0.25 mg/ml) were used to substitute for recombinant TFIIA, rTFIE/rTFIF-FLAG-tagged TFIIH and PC4, respectively, in the transcription assays. Reactions were then performed and analyzed as described (Chiang et al., 1993). The transcription signals were quantitated by PhosphorImager (Molecular Dynamics).

Acknowledgements

We are grateful to I.Davidson for anti-TAF28 antibodies, J.-M.Egly for anti-p34, -p44 and -p52 antibodies, E.Golemis for anti-RPB4 and -RBP7 antibodies, D.Morgan for anti-cdk7, -cycH and -MAT1 antibodies, D.Reinberg for pG5MLT and anti-p89 and -p80 antibodies, R.Groeder for anti-TBP, -TAF11, -p62, -RBP6 and -SRB7 antibodies, N.Thompson and R.Burgess for anti-RPB1, -RBP2 and -RBP8 antibodies, and E.Lees and R.Burgess for anti-RPB1, -RPB2 and -RPB8 antibodies. We also thank E.Golemis for information on RPB4 and RPB7, and R.Dodson and D.Shapiro for comments on the manuscript. C.-M.C. is a Pew Scholar in the Biomedical Sciences. This research is supported in part by Research Grant # 5-FY96-1196 from the March of Dimes Birth Defects Foundation and in part by Research Project Grant # RPG-97-135-01-MBC from the American Cancer Society.

References

Acker,J., de Graaff,F., Cheynel,I., Khazak,V., and R.Dodson and D.Shapiro for comments on the manuscript. C.-M.C. is a Pew Scholar in the Biomedical Sciences. This research is supported in part by Research Grant # 5-FY96-1196 from the March of Dimes Birth Defects Foundation and in part by Research Project Grant # RPG-97-135-01-MBC from the American Cancer Society.


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