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Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerases II and III

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Two multisubunit complexes containing the TATA-binding protein (TBP) were isolated from HeLa cells constitutively expressing the FLAG epitope-tagged TBP using antibody affinity and peptide elution methods. One of the complexes (f:TFIID), isolated from the P11 0.85 M KCl fraction, contains at least 13 specific TBP-associated factors (TAFs) and can mediate activator-dependent transcription by RNA polymerase II. Importantly, activator function through the highly purified f:TFIID complex still requires a general cofactor fraction containing upstream factor stimulatory activity (USA). As previously observed with partially purified activator-competent natural TFIID, f:TFIID generates extended TATA-dependent footprints on the intrinsically strong adenovirus major late promoter (MLP) but only restricted footprints on the weak adenovirus E1b and E4 and HIV (core) promoters. Along with previous demonstrations of activator-induced downstream TFIID interactions on the E4 promoter, these results argue for a relationship between downstream interactions and overall promoter strength. Initiator-like sequences appear not to be essential for downstream interactions since they have no effect on downstream MLP interactions when mutated, do not effect downstream interactions on the HIV promoter and are not present on the inducible E4 promoter. The other multisubunit complex (f:TFIIIB), isolated from the P11 0.30 M KCl fraction, contains four specific TAFs and can substitute for one of the fractions (TFIIIB) required for RNA polymerase III (pol III) transcription. Neither f:TFIID nor TBP could substitute for this pol III TBP-containing fraction. This plus the fact that f:TFIIIB failed to generate a footprint on the MLP underscores the importance of TAFs in determining promoter specificity by different RNA polymerases.

Key words: epitope-tagged TBP/TAF/TFIID/TFIIIB/transcription

Introduction

Genes transcribed by nuclear RNA polymerases I, II and III have distinct promoter elements that serve as binding sites for gene-specific regulators and for basal transcription initiation factors unique to each RNA polymerase. Interactions between these various accessory factors then recruit specific RNA polymerases to the promoter regions for initiation of transcription. For the mRNA-encoding class II genes, an array of basal transcription factors is first assembled on core promoters which typically contain a TATA box upstream of the transcription initiation site and/or an initiator (Inr) element at or near the start site. These core promoter factors include TFIIA, -B, -D, -E, -F, -G/J, -H and -I, and have been chromatographically separated, purified and, in several cases, cloned (for reviews, see Roeder, 1991; Zawel and Reinberg, 1992).

TFIID was first identified as a general initiation factor for RNA polymerase II (Matsui *et al.*, 1980) that binds directly to the TATA box (Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988). On TATA-containing promoters transcribed by RNA polymerase II, the commonly studied pathway involves primary TATA recognition by TFIID with subsequent ordered interactions of the remaining factors to form a functional preinitiation complex (Van Dyke *et al.*, 1988; Buratowski *et al.*, 1989; Roeder, 1991). Consistent with its central role in recruiting other basal factors to the promoter, TFIID was also implicated in transcriptional activation by the demonstration of physical and functional interactions between TFIID and various cellular and viral activators, including USF (Sawadogo and Roeder, 1985), ATF (Hai *et al.*, 1988; Horikoshi *et al.*, 1988b), Gal4 (Horikoshi *et al.*, 1988a) and the pseudorabies IE protein (Abmayr *et al.*, 1988; Workman *et al.*, 1988). Although further studies of this critical initiation factor from mammalian cells were hampered by its instability, large molecular mass, heterogeneous chromatographic behavior and low yields during purification (Reinberg and Roeder, 1987; Nakajima *et al.*, 1988), the identification, purification and cloning of a small yeast TATA-binding protein (TBP) that could substitute for TFIID in basal transcription (Buratowski *et al.*, 1988; Cavallini *et al.*, 1989; Eisenmann *et al.*, 1989; Hahn *et al.*, 1989a; Horikoshi *et al.*, 1989a,b; Schmidt *et al.*, 1989; for reviews, see Greenblatt, 1991; Pugh and Tjian, 1992; Sharp, 1992) led ultimately to the cloning of cDNAs encoding homologous proteins in higher eukaryotes including *Drosophila* (Hoey *et al.*, 1990; Muhich *et al.*, 1990) and human (Hoffmann *et al.*, 1990; Kao *et al.*, 1990; Peterson *et al.*, 1990). Consistent with earlier studies with TFIID, TBP was found to interact with various transcription regulators including VP16 (Stringer *et al.*, 1990; Ingles *et al.*, 1991), Zta (Lieberman and Berk, 1991), E1A (Ptashne and Gann, 1990; Horikoshi *et al.*, 1991; Lee *et al.*, 1991), T-antigen (Gruda *et al.*, 1993) and p53 (Seto *et al.*, 1992; Truant *et al.*, 1993). However, the much smaller size (38 kDa) and restricted DNA binding properties (interactions only over the TATA box) of human and *Drosophila* TBPs relative to the much larger sizes (~700 kDa; Conaway *et al.*, 1991; Timmers and Sharp, 1991) and extended DNA binding properties on some promoters (Parker and Topol, 1984; Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988; Nakatani *et al.*, 1990) exhibited by the corresponding natural TFIID species indicated that the latter are multisubunit complexes. It was further observed

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that while native TFIID mediates both basal and activator-dependent transcription (Sawadogo and Roeder, 1985; Horikoshi *et al.*, 1988b; Nakajima *et al.*, 1988), TBP supports only basal transcription in more purified reconstituted systems (Hoffmann *et al.*, 1990; Peterson *et al.*, 1990; Pugh and Tjian, 1990). Taken together, these results indicated that the TBP-associated factors (TAFs) in native TFIID are essential for the activator-mediated transcription.

Recent studies utilizing immunoaffinity approaches have resulted in the identification of TAFs from both *Drosophila* (Dynlacht *et al.*, 1991) and human cells (Pugh and Tjian, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992; Zhou *et al.*, 1992). The chromatographic fractions containing these polypeptides have been shown to mediate activator function in conjunction with crude fractions containing TBP (Dynlacht *et al.*, 1991; Brou *et al.*, 1993a,b). These observations are consistent with the presumed functions of the TAFs, whose complexity raises the interesting possibility that different TAFs may be selective for distinct regulatory factors. Interestingly, transcription from TATA-less class II promoters also requires a multisubunit TFIID complex (Pugh and Tjian, 1990, 1991; Smale *et al.*, 1990). Other related studies have identified a novel class of distinct cofactors that dramatically enhance induction by gene-specific activators (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991; Inostroza *et al.*, 1992). These cofactors [e.g. upstream factor stimulatory activity (USA)] include both negative components which repress basal (activator-independent) promoter activity and positive components which enhance activator function.

The synthesis of many small structural RNA molecules is carried out by RNA polymerase III (see reviews by Geiduschek and Tocchini-Valentini, 1988; Kunkel, 1991). The corresponding promoter elements may be intragenic or extragenic and interact with both common and gene-specific factors. The general transcription factors TFIIIB and TFIIIC are necessary for assembly of functional preinitiation complexes on tRNA and VA_I RNA genes, while TFIIIA is required, in addition, for 5S RNA genes (Lassar *et al.*, 1983). The stable binding of TFIIIC (VA_I and tRNA) or TFIIIA plus TFIIIC (5S RNA) to these promoters results in the recruitment of TFIIIB, which is the transcription initiation factor proper that alone correctly positions RNA polymerase III for repeated cycles of transcription (Kassavetis *et al.*, 1990). This property of TFIIIB is reminiscent of that exhibited by TFIID (Hawley and Roeder, 1987; Van Dyke *et al.*, 1988) in higher eukaryotes. Interestingly, recent studies also have indicated that TBP is required for transcription by RNA polymerase III and is present within the TFIIIB fraction (Kassavetis *et al.*, 1992; Lobo *et al.*, 1992; Simmen *et al.*, 1992a; Taggart *et al.*, 1992; White and Jackson, 1992; reviewed by Rigby, 1993). Furthermore, TBP and some associated factors that are distinct from those TAFs identified in native TFIID complexes were also shown to be integral components of the RNA polymerase I selectivity transcription factor (SL1) (Comai *et al.*, 1992). These results indicated that the promoter selectivity among different RNA polymerases may be determined in part by TAFs that associate with TBP.

The evolutionary conservation of transcription processes by nuclear RNA polymerases I, II and III is further exemplified by the recent identification of TFIIB-related factors (within TFIIIB) involved in transcription by RNA polymerase III (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-De-León *et al.*, 1992). Moreover, TFIIA has also been reported to be required for *in vitro* transcription of mammalian U6 genes by RNA polymerase III (Waldschmidt and Seifart, 1992). Therefore, it seems that utilization of common transcription initiation factors, such as TBP, by all three RNA polymerases is more delicately regulated at the level of transcription initiation by processes that must involve additional gene and/or polymerase specific factors.

To identify these TBP-containing complexes involved in transcription by RNA polymerases II and III, we have employed an efficient epitope-tagging and purification method that utilizes commercially available antibodies and affinity resins. The purified complexes (TFIID and TFIIIB) contain different polypeptide compositions, more complex than previously identified by related affinity purification methods (Pugh and Tjian, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992; Zhou *et al.*, 1992), and show differential activities in transcription by RNA polymerases II and III as well as different properties in physical DNA binding assays. Moreover, in the case of transcription by RNA polymerase II, and despite the complexity intrinsic to the affinity purified TBP complexes, a high level of activator-dependent transcription still requires additional cofactors (USA) not essential for basal transcription.

Results

Epitope-tagged TBP shows nuclear localization in transfected cells

A synthetic, 8 amino acid FLAG epitope (Hopp *et al.*, 1988; Prickett *et al.*, 1989) has been used successfully to express a variety of tagged proteins in bacteria and yeast. This expression system is further facilitated by commercially available monoclonal antibodies and antibody-conjugated affinity resins used for protein detection and purification. To purify multisubunit TBP-containing protein complexes from mammalian cells using the epitope-tagging method, we first constructed two human TBP expression plasmids utilizing the FLAG epitope, either alone or in combination with the influenza hemagglutinin (HA) epitope (Wilson *et al.*, 1984; Field *et al.*, 1988; Kolodziej and Young, 1991). The feasibility of the HA epitope for purifying multisubunit protein complexes had been demonstrated for yeast adenylyl cyclase (Field *et al.*, 1988), yeast RNA polymerase II (Kolodziej *et al.*, 1990) and human TFIID (Zhou *et al.*, 1992). Expression of the FLAG-tagged TBP (f:TBP) and HA/FLAG-tagged TBP (hf:TBP) was first monitored in transiently transfected COS-7 cells by immunofluorescence. Both f:TBP and hf:TBP showed intense nuclear staining signals when anti-FLAG M2 monoclonal antibodies were used (Figure 1B and C). No signal was detected when cells were transfected with the TBP expression plasmid, due to the lack of the FLAG epitope, or when anti-E1A monoclonal antibodies were used (Figure 1A and D). The immunoreactivities and sizes of the epitope-tagged proteins were also examined following expression, along with the untagged (TBP) and 6-histidine-tagged (6His:TBP) proteins, in bacteria (Studier *et al.*, 1990). Only f:TBP and hf:TBP, but not 6His:TBP or TBP, were detected by Western blotting when anti-FLAG M2 monoclonal antibodies were used

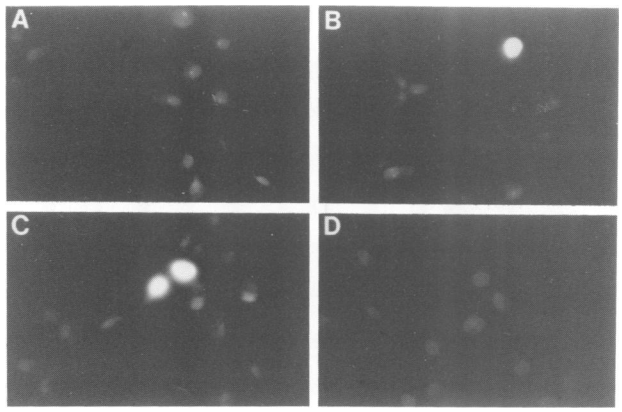

Fig. 1. Nuclear localization of the epitope-tagged TBP in transiently transfected COS-7 cells. COS-7 cells were transfected by pMT2-TBP (**A**), pMT2-F:TBP (**B**) and pMT2-HF:TBP (**C** and **D**) expressing TBP, f:TBP and hf:TBP, respectively, in two-well chambered slides as described in Materials and methods. The anti-FLAG M2 monoclonal antibody (A, B and C) or the anti-E1A monoclonal antibody (D) were used as the primary antibody for indirect immunofluorescence staining.

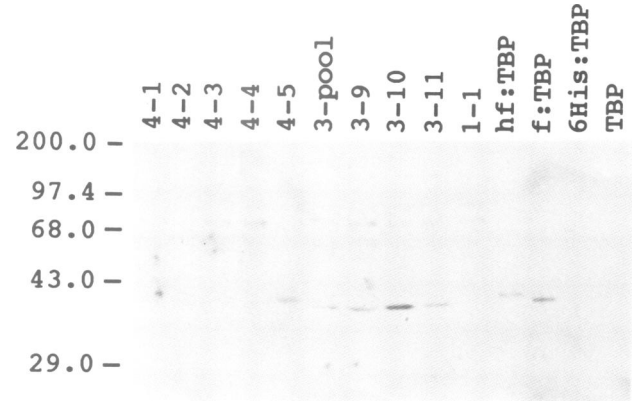

Fig. 2. Expression of the epitope-tagged TBP in different HeLa-derived cell lines as revealed by Western blotting using the anti-FLAG M2 monoclonal antibody. Individual G418-resistant HeLa cell lines were established by retrovirus-mediated gene transfer expressing hf:TBP (4-1, 4-2, 4-3, 4-4 and 4-5) and f:TBP (3-9, 3-10 and 3-11). The cell line 3-pool was established from pooled G418-resistant colonies expressing f:TBP. 1-1 is a control cell line containing only the expression vector without the insert. Bacterial lysates containing separately expressed hf:TBP, f:TBP, 6His:TBP and TBP were included for comparison. Sizes of the protein markers (in kDa) are indicated on the left of the gel.

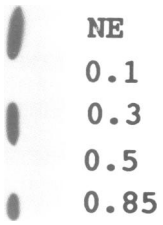

Fig. 3. Distribution of f:TBP in different P11 fractions revealed by Western blotting using the anti-FLAG M2 monoclonal antibody. Nuclear extracts (NE) of 3-10 cells were fractionated on a P11 phosphocellulose column using different molar concentrations of KCl as indicated.

(Figure 2). All these TBP-derived proteins were observed by using anti-TBP polyclonal antibodies (data not shown). These results indicate that the correctly expressed epitope-tagged TBP has the localization expected for a nuclear transcription factor. Furthermore, it shows the specificity of the FLAG epitope in mammalian cells, since no major cross-reacting signals were observed. Interestingly, a unique nucleolar staining signal was not seen as might be expected from active RNA polymerase I transcription.

Constitutive expression of the epitope-tagged TBP in HeLa cells

To establish HeLa cell lines constitutively expressing the epitope-tagged TBP for purification of multisubunit TBP complexes, we employed the retrovirus-mediated gene transfer technique which has been used successfully for stable cell line selection and recovery of rare viral cDNAs (Chiang *et al.*, 1991). All established HeLa cell lines express different levels of the epitope-tagged TBP, as revealed by Western blotting using anti-FLAG M2 monoclonal antibodies (Figure 2). This is probably due to integration of retroviral DNA sequences at random chromosomal sites. When anti-TBP antibodies were used, these cell lines showed similar levels of TBP expression (data not shown), consistent with previous observations (Zhou *et al.*, 1992). This result suggests that regulated expression of TBP is essential for cell growth. To evaluate possible cytotoxic effects of TBP, we cotransfected the TBP expression plasmid with a G418 selection marker at a 10:1 ratio and scored drug-resistant colonies after a 3-week selection (Chiang *et al.*, 1992). HeLa cells transfected with TBP expression plasmids driven by the adenovirus major late promoter showed at least 5- to 10-fold fewer colonies than other HeLa cells transfected with control plasmids (data not shown). This result further underscores the relevance of tightly regulated TBP expression in mammalian cells.

Compositions of TBP-associated factors in P11 0.3 M and 0.85 M KCl fractions

To purify TBP complexes, nuclear extracts from 3-9, 3-10, 4-5 (see Figure 2) and HeLa cells were resolved into four previously characterized fractions (Meisterernst *et al.*, 1991) by sequential salt elution (0.1, 0.3, 0.5 and 0.85 M KCl) from phosphocellulose (P11) columns. The distribution of TBP across the P11 fractions, as detected by Western blotting using anti-FLAG M2 or anti-TBP antibodies, was similar in all four cell lines (Figure 3 and data not shown). TBP is found primarily in the 0.30 M and 0.85 M KCl fractions with relative levels of around 60% and 40%, respectively, in good agreement with previous results (Timmers and Sharp, 1991). The TBP complexes in the nuclear extract, P11 0.30 M (P.30) and 0.85 M (P.85) KCl fractions were then isolated from 3-10 cells using the anti-FLAG M2 monoclonal antibody-conjugated agarose (see Materials and methods). Corresponding P11 fractions from HeLa cells were also analyzed in parallel and served as controls for the affinity purification. Epitope-tagged TBP-containing complexes isolated from P.30 (f:P.30) and P.85 (f:P.85) show distinct polypeptide compositions (Figure 4, lanes 3–6). Complexes with the same polypeptides could also be isolated directly from unfractionated nuclear extracts (Figure 4, lanes 1 and 2), although more non-specific proteins were detected.

The compositions of TAFs in f:P.30 and f:P.85 are related to but more complex than those previously reported for

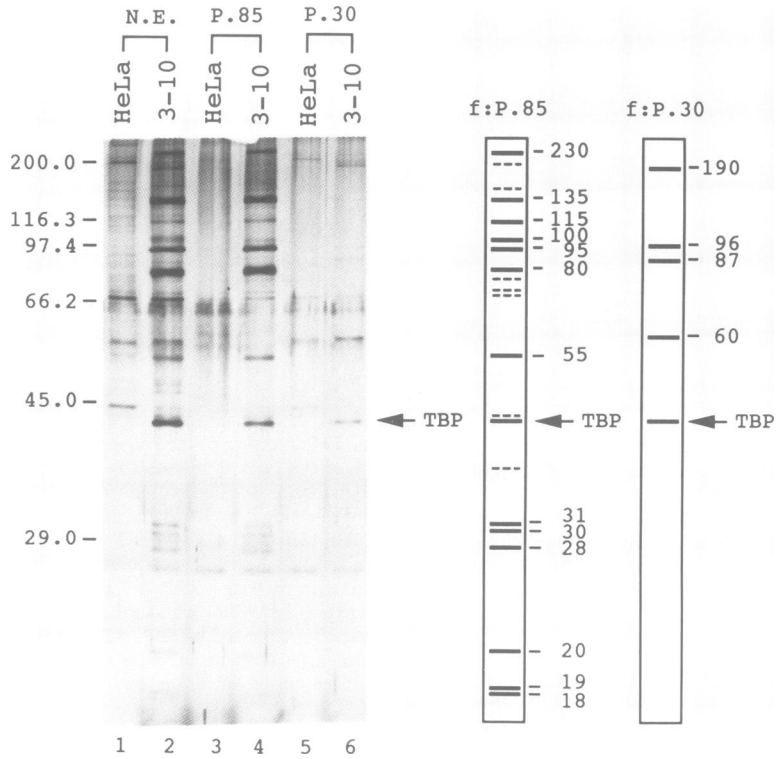

Fig. 4. Polypeptide compositions of f:TBP complexes isolated from different P11 fractions. Affinity-purified complexes from nuclear extracts (N.E.), P11 0.85 M KCl fractions (P.85) and P11 0.30 M KCl fractions (P.30) of HeLa (lanes 1, 3 and 5) and 3-10 (lanes 2, 4 and 6) were separated on a 10% polyacrylamide–SDS gel and visualized by silver staining. Unique polypeptides present in 3-10 but not HeLa are summarized on the right using solid lines. Less definitively specific bands are depicted by dashed lines. Sizes of protein markers are indicated on the left of the gel and predicted sizes of associated polypeptides are indicated on the right in kDa. The lower ratio of TBP in the f:P.30 and f:P.85 preparations, relative to the ratio in the P.30 and P.85 fractions (Figure 3), reflects a lower input of f:P.30 (based on cellular equivalents) in the present analysis.

B-TFIID (Timmers *et al.*, 1992) and for an epitope-tagged affinity purified TFIID (Zhou *et al.*, 1992), which were derived from fractions similar to P.30 and P.85, respectively. The f:P.85 contains five major TAFs with relative molecular weights around 230, 135, 95, 80 and 55 kDa, which are probably equivalent to the major TAFs (250, 125, 95, 78 and 50 kDa) detected by Berk and colleagues (Zhou *et al.*, 1992). Interestingly, under our washing conditions (0.3 M KCl plus 0.1% Nonidet P-40), we also detected several minor but specifically retained TAFs (115, 110, 31, 30, 28, 20, 19 and 18 kDa) that had not been clearly seen before (Pugh and Tjian, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992; Zhou *et al.*, 1992). However, some of these were also evident in the anti-TBP antibody selected TFIID preparations (Pugh and Tjian, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992). The smaller TAFs may be present in roughly stoichiometric amounts. Additional associated minor polypeptides that might, however, be present (at quantitatively reduced levels) in the control HeLa fractions are also indicated (dashed lines in Figure 4 summary, also see Figure 5). In f:P.30, we detected three major TAFs (190, 87 and 60 kDa) that are clearly different from those present in f:P.85 (Figure 4), as well as a 96 kDa TAF whose relationship to the 95 kDa polypeptide of f:P.85 remains to be investigated. The 60 kDa TAF present in f:P.30 migrates slower than a less abundant, non-specific polypeptide which is also seen in the control fraction, presumably as a result of interactions with the agarose portion of the affinity resin.

To determine whether the expression level of f:TBP and/or different epitope tags might affect the composition of the

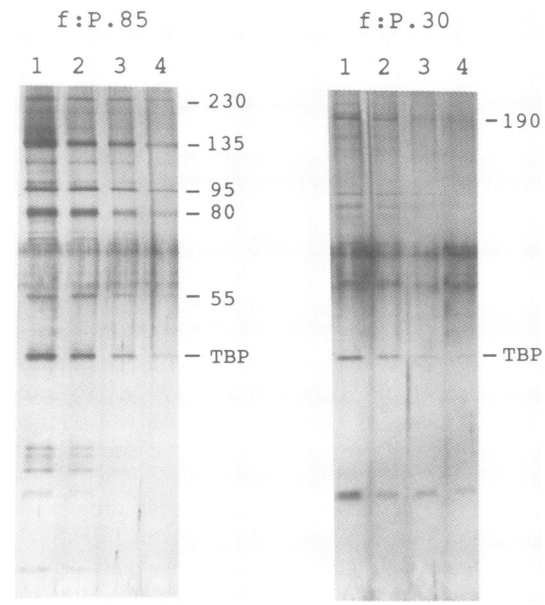

Fig. 5. Polypeptide compositions of sequentially eluted f:TBP complexes. Serial elutions of complexes from either P11 0.85 M KCl (f:P.85) or 0.30 M KCl (f:P.30) are shown on a silver-staining gel with estimated molecular sizes (in kDa) of some proteins indicated on the right of the gel.

complex, f:P.30 and f:P.85 were also isolated from 3-9 cells, which express lower amounts of f:TBP, and from 4-5 cells, which express the dual HA/FLAG-tagged TBP (see

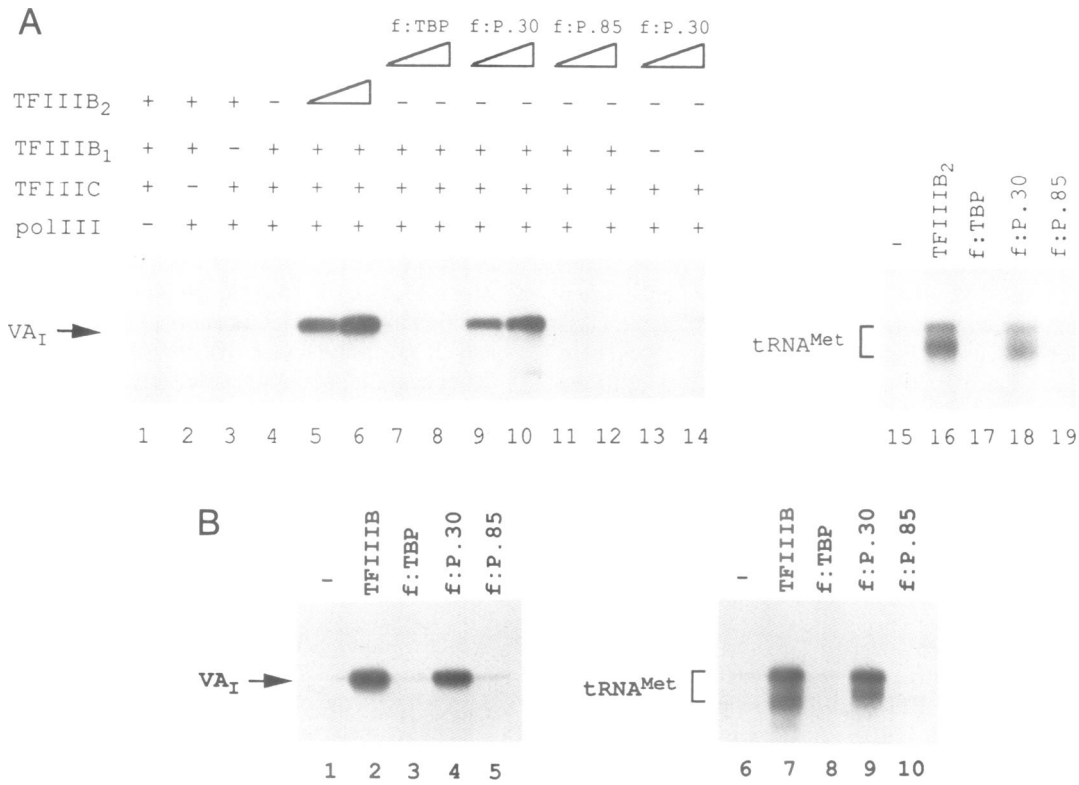

Fig. 6. Substitution of the f:P.30 complex for a TFIIIB subfraction and for TFIIIB in mediating RNA polymerase III transcription. (**A**) f:P.30 is functionally equivalent to $TFIIIB_2$ in mediating pol III-transcription. The transcription reactions were reconstituted with 0.1 μl of RNA polymerase III (10 units, 0.025 mg/ml), 2 μl of TFIIIC (0.035 mg/ml), 2 μl of $TFIIIB_1$ (0.5 mg/ml) and 2 μl of $TFIIIB_2$ (0.34 mg/ml) where indicated (lanes 1−4, +). Increasing amounts of the tested components were added as follows: 1 μl of $TFIIIB_2$ (lane 5), 2 μl of $TFIIIB_2$ (lanes 6 and 16), 5 ng of f:TBP (lanes 7 and 17), 10 ng of f:TBP (lane 8), 1 μl of f:P.30 diluted 5-fold in BC100 plus 0.5 mg/ml of BSA (lanes 9 and 13), 2 μl of the above diluted f:P.30 (lanes 10, 14 and 18), 1 μl of f:P.85 diluted 5-fold in BC100 plus 0.5 mg/ml of BSA (lane 11), 2 μl of the above diluted f:P.85 (lanes 12 and 19). (**B**) Substitution of f:P.30 for TFIIIB in pol III-transcription. Transcription reactions were performed as described above (Figure 6A, lanes 15−19) except that 2 μl of the P11 TFIIIC fraction was used instead of the 3-column purified TFIIIC and no $TFIIIB_1$ and $TFIIIB_2$ were added in the reaction.

Figure 2). No difference in TAF compositions was detected by silver staining (data not shown). Therefore, f:P.30 and f:P.85 complexes were isolated from 3-10 cells on a preparative scale (see Materials and methods) and taken as being of standard composition and general relevance. To determine the efficiency of peptide elution, we reincubated the eluted resins with a fresh peptide solution for a total of four times. Serial 20 min elutions of f:P.85 and f:P.30 from the M2-agarose showed progressively reduced levels of the TAFs (Figure 5). Interestingly, the fourth elution of f:P.85 and the third elution of f:P.30 exhibited TAF compositions (indicated on the right of each panel) similar to those previously detected for TFIID (Zhou *et al.*, 1992) and B-TFIID (Timmers *et al.*, 1992), respectively. The apparent differences between the early and late eluting fractions may reflect heterogeneous populations of TBP-containing complexes with differential affinities for the FLAG antibody. Alternatively, they may reflect lower recoveries of residual but identical complexes coupled with a relatively insensitive assay. Regardless of the actual situation, these analyses have detected additional TBP-associated polypeptides that may not have been detected in previous assays for related reasons. The efficiency of recovery in an initial elution could be enhanced to >70% by extending the incubation time with the peptide solution to 1 h (data not shown). The first elutions of each complex as in Figure 5 were used for the following transcription studies and binding assays.

f:P.30 can substitute for one of the components required for RNA polymerase III transcription in vitro

The P11 0.3 M KCl fraction has long been known to contain the general RNA polymerase III initiation factor TFIIIB (Segall *et al.*, 1980) and recent studies have established that TBP is an essential component of this complex (Kassavetis *et al.*, 1992; Lobo *et al.*, 1992; Simmen *et al.*, 1992a; Taggart *et al.*, 1992; White and Jackson, 1992). A more extended purification of a TBP-containing complex from this same fraction yielded an activity (B-TFIID) that could mediate basal, but not activator-dependent, transcription by RNA polymerase II (Timmers and Sharp, 1991; Timmers *et al.*, 1992). However, the exact relationship between B-TFIID and the TBP-containing TFIIIB complex is not clear. To determine if f:P.30 is functionally equivalent to TFIIIB or one of the basic components required for transcription by RNA polymerase III, we developed a reconstituted TBP-dependent RNA polymerase III transcription system (see Materials and methods). In this system, the classical (P11 0.30 M KCl) TFIIIB fraction (Segall *et al.*, 1980) was further fractionated into two components on a Mono Q column (see Materials and methods). The fraction eluting at 0.21 M KCl ($TFIIIB_1$) contains no TBP, while the fraction eluting at 0.28 M KCl ($TFIIIB_2$) contains TBP as determined by Western blotting using anti-TBP antibodies (data not shown). As shown in Figure 6A, both $TFIIIB_1$ and $TFIIIB_2$ components are required, in conjunction with

a highly purified TFIIIC, for transcription of adenovirus VA_I RNA by RNA polymerase III (lanes 1–4 versus 5 and 6). The low level of transcription detected in the absence of $TFIIIB_1$ (lane 3) is probably due to a minor $TFIIIB_1$ contaminant in other components (see below). In this reconstituted system, f:P.30 (lanes 9 and 10) could effectively substitute for $TFIIIB_2$ (lanes 5 and 6), whereas f:P.85 (lanes 11 and 12) and TBP (lanes 7 and 8) could not, and showed the same dependence on $TFIIIB_1$ (lanes 9 and 10 versus 13 and 14). Similar results were obtained when a tRNA template containing a comparable intragenic class III promoter was used (Figure 6A, lanes 15–19). These results suggest that f:P.30 is functionally equivalent to the $TFIIIB_2$ involved in transcription by RNA polymerase III. Interestingly, when a TFIIIB-dependent transcription system using the same RNA polymerase III but a less purified TFIIIC (P11 0.60 M KCl) fraction was used, f:P.30 alone (without $TFIIIB_1$) was sufficient to restore transcription of both VA_I and tRNA templates (Figure 6B, lanes 2, 4, 7 and 9). As expected, TBP and f:P.85 could not substitute for TFIIIB in RNA polymerase III-mediated transcription (Figure 6B, lanes 3, 5, 8 and 10). This result suggests that f:P.30 is equivalent to the conventionally defined TFIIIB (or $TFIIIB_2$) and that the P11 TFIIIC fraction contains the functional equivalent of $TFIIIB_1$ fraction in addition to the well defined five-subunit TFIIIC (Kovelman and Roeder, 1992). The use of partially purified TFIIIC preparations may explain why other investigators (Lobo *et al.*, 1992; Simmen *et al.*, 1992a) failed to detect the $TFIIIB_1$ activity that elutes from the Mono Q column at 0.21 M KCl. Lobo *et al.* (1992) separated the TFIIIB fraction into a TBP-containing activity eluting at 0.38 M KCl and a TBP-free activity eluting at 0.48 M KCl from the Mono Q column. These subfractions could reflect derivatives from a component similar to our $TFIIIB_2$ on the basis of the protein compositions (190, 96, 87 and 60 kDa in our $TFIIIB_2$ versus 150, 82 and 54 kDa in their 0.38 M KCl fraction) or they could reflect the separation of a distinct RNA polymerase III activity from a similar $TFIIIB_2$ component.

f:P.85 can substitute for partially purified TFIID in mediating activator-dependent transcription by RNA polymerase II but still requires the general cofactor fraction USA

TFIID elutes from the phosphocellulose column in the 0.85 M KCl fraction (Matsui *et al.*, 1980; Nakajima *et al.*, 1988). To test if f:P.85 is functionally equivalent to the previously characterized TFIID, we used a reconstituted *in vitro* transcription system that is dependent upon both a general cofactor fraction (USA) and native TFIID, in addition to other general factors (TFIIA, TFIIB, TFIIE/F/H and RNA polymerase II) for high levels of activator-dependent transcription (Meisterernst *et al.*, 1991). The template employed for this analysis was pG_5HMC_2AT, which contains five GAL4 binding sites preceding the HIV TATA box and the MLP initiator element linked to a G-less cassette of 380 bp. The template pMLΔ53, containing the adenovirus major late promoter without the upstream USF binding site, was used as an internal control. The analysis used equivalent molar amounts of f:TBP, f:P.30 and f:P.85 which were normalized on the basis of TBP content by Western blotting (data not shown). As shown in Figure 7, in the absence of TFIID, no specific transcription could be detected with the other basal factors alone or in combination with either the gene-specific activator GAL4-AH or the USA fraction (lanes 1–3). A low level of transcription was detected when both GAL4-AH and USA were added (lane 4), probably due to trace amounts of TFIID contamination in the partially purified general factor or USA fractions. This is exactly reminiscent of the situation in other less well defined TFIID-deficient systems in which low levels of activator-dependent transcription (above undetectable basal) were always observed in the absence of exogenous TFIID (cf. Tanese *et al.*, 1991; Zhou *et al.*, 1992).

In the presence of a partially purified TFIID (Mono S) fraction from HeLa nuclear extracts, a low level of basal transcription was observed (lane 5). Under these conditions, GAL4-AH markedly enhanced transcription from the HIV promoter in the presence (lane 8), but not in the absence

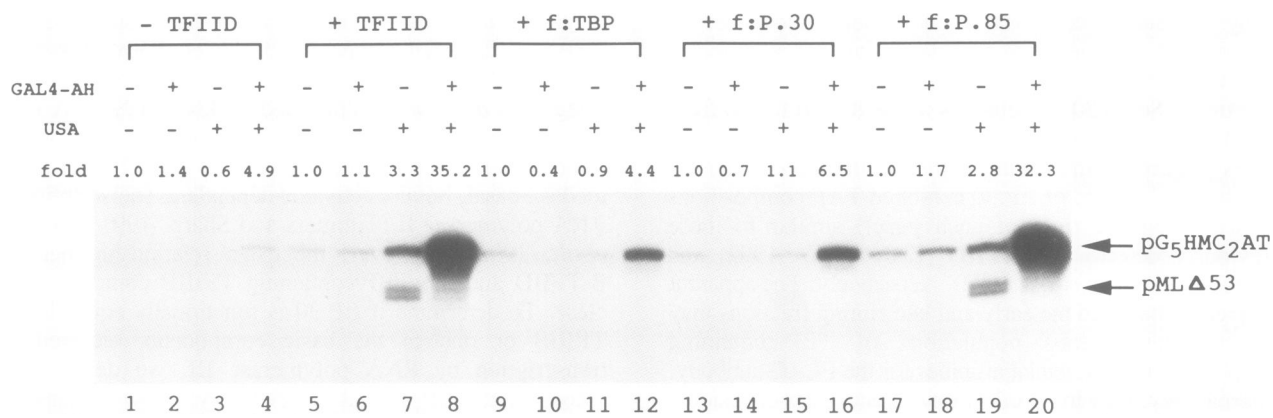

Fig. 7. Requirement of TFIID or f:P.85 for USA-mediated transcriptional activation by GAL4-AH. *In vitro* transcription from the HIV promoter (pG_5HMC_2AT) and the adenovirus major late core promoter (pMLΔ53) was reconstituted with recombinant or partially purified general factors in the presence (+) or absence (−) of GAL4-AH (50 ng) or USA (2 μl, 0.28 mg/ml) as described in Materials and methods. Equivalent amounts of f:TBP, f:P.30, f:P.85 (quantified by TBP concentration using Western blotting) and partially purified TFIID were used in the reactions where indicated (+). Numbers immediately above the lanes represent the activation (fold) in the presence of GAL4-AH and/or USA, measured by liquid scintillation counting of the relevant slices of dried gels.

(lane 6) of USA. Consistent with previous studies (Meisterernst *et al.*, 1991), a part of this enhancement could be attributed to basal effects of USA, apparently in conjunction with the contaminating TFIID (lane 7; note also the effect of USA on the core MLP control). Qualitatively and quantitatively similar activator- and USA-dependent effects on activation of the HIV promoter were observed when f:P.85 was used in place of the partially purified TFIID (compare lanes 17–20 with lanes 5–8). TBP and f:P.30 behaved like TFIID and f:P.85 in that each brought about a low level of basal activity but showed no activation by GAL4-AH alone (lanes 9 versus 10 and 13 versus 14). However, in contrast to TFIID and f:P.85, TBP and f:P.30 effected no response to USA and only a weak activation in response to both USA and GAL4-AH (lanes 11 versus 12 and 15 versus 16). As mentioned above, the low levels of activation that are apparent with GAL4-AH and USA appear to be due to minor TFIID contamination in partially purified general factor or USA fractions that may be slightly enhanced by TBP-containing fractions (e.g. by titration of endogenous negative cofactors, cf. Meisterernst *et al.*, 1991). Results similar to those described here with GAL4-AH were also obtained when Sp1 was employed as an activator in conjunction with the wild-type HIV promoter (data not shown). Therefore, f:P.85 is functionally equivalent to TFIID, in terms of both basal and activator-mediated transcription, whereas f:P.30 supports only basal transcription in this reconstituted system. Of special note, however, is the fact that in this more purified system, the highly purified but nonetheless structurally complex epitope-tagged TFIID still requires the USA cofactor constituents for efficient activator-dependent transcription.

f:P.85, like TFIID, generates downstream footprints on the adenovirus major late promoter

Another inherent property of human TFIID is the extensive downstream interactions (revealed by DNase I footprints) on the adenovirus major late (Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988) and human glial fibrillary acidic protein (Nakatani *et al.*, 1990) promoters, in contrast to the restricted interactions over the TATA region shown by yeast or human TBP (Hahn *et al.*, 1989b; Horikoshi *et al.*, 1989b; Kao *et al.*, 1990; Peterson *et al.*, 1990). To test if f:P.85 would show equivalent MLP interactions, we performed DNase I footprinting on both non-transcribed and transcribed DNA strands. As expected, f:P.85 revealed extended footprints and hypersensitive sites (over the region −40 to +35) indistinguishable from those typically observed with

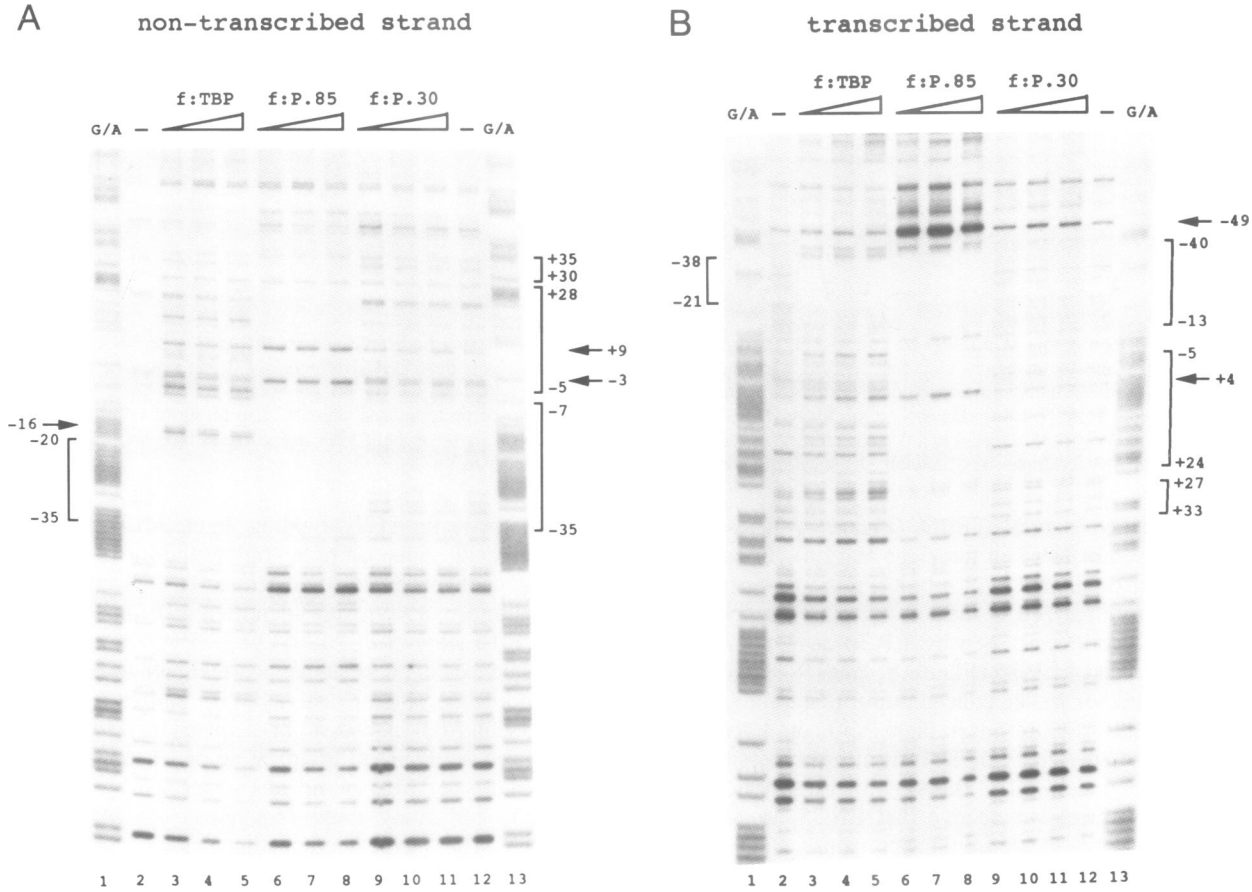

Fig. 8. DNase I footprinting of f:TBP complexes on the adenovirus major late promoter. The non-transcribed (**A**) and transcribed (**B**) DNA templates spanning −91 to +85 of the major late promoter were prepared as described in Materials and methods. The Maxam–Gilbert sequencing method (Maniatis *et al.*, 1982) was used to prepare the G/A footprinting marker (lanes 1 and 13). No protein (lanes 2 and 12) or increasing amounts of f:TBP (12 ng, 25 ng and 50 ng for lanes 3–5), f:P.85 (0.5, 1 and 2 μl for lanes 6–8), or f:P.30 (0.5, 1 and 2 μl for lanes 9–11) were included in the binding reaction. The protected regions are marked by brackets and the enhanced DNase I cleavage sites are indicated by arrows.

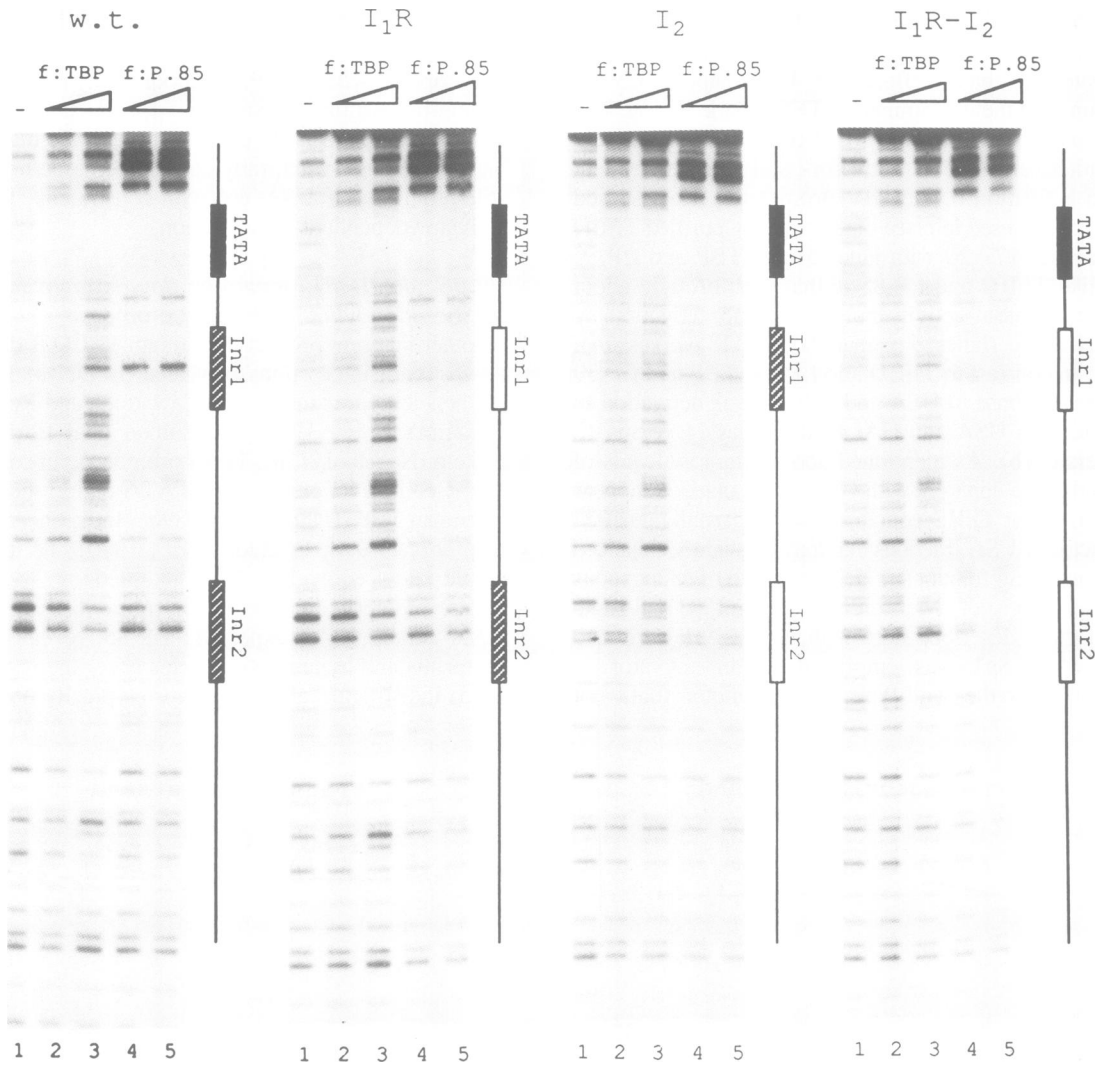

Fig. 9. DNase I footprinting of f:TFIID on core promoter fragments with wild-type or mutated initiator elements. Preparation of DNA templates with wild-type or initiator mutations including −45 to +65 major late promoter sequences is described in Materials and methods. The I_1R, I_2 and I_1R-I_2 templates contain mutations in positions +3 to +5 of Inr 1, in positions +42, +46, +48, +53, +54, +56 and +57 of Inr 2, and in the combined positions from both Inr elements, respectively (Du *et al.*, 1993). The amounts of proteins used were: 12 and 50 ng of f:TBP (lanes 2 and 3), 0.5 and 2.0 μl of f:P.85 (lanes 4 and 5). No protein was included in lanes 1. The locations of the TATA region (solid box) and the wild-type (hatched box) or mutated (open box) initiator elements are indicated on the right of each panel.

partially purified natural TFIID (Figure 8, lanes 6−8; interactions summarized in the right margin). In contrast, f:TBP protected only the TATA box (Figure 8, lanes 3−5; interactions summarized in the left margin). Surprisingly, no clear protection was seen by f:P.30 at a molar input equivalent to that of f:P.85 (Figure 8, lanes 9−11) or even at a 10-fold higher molar concentration (data not shown), indicating that TAFs present in the f:P.30 could divert the TATA-binding specificity of TBP from promoters utilized by RNA polymerase II. Based on transcription and footprinting studies, we believe that f:P.85 has intrinsic properties indistinguishable from the previously characterized TFIID. Henceforth, f:TFIID will be used for f:P.85 to specify the TFIID purified by the FLAG epitope-tagging method.

Mutations in the initiator regions have no qualitative effect on f:TFIID binding to the major late promoter

The extended downstream footprints observed on the MLP indicate the potential involvement of downstream initiator (Inr) elements for TFIID binding. In the MLP, two Inr-like elements with sequence homology to the Inr of the murine terminal deoxynucleotidyl transferase (TdT) promoter (Smale and Baltimore, 1989) were identified and found to interact with TFII-I (Roy *et al.*, 1991) or USF[43] (Du *et al.*, 1993) and sequences in these elements are known to be important for optimal promoter activity. To address a potential contribution from individual Inr elements for TFIID binding, we carried out DNase I footprinting on several mutated Inr (and wild-type) MLP templates generated by PCR using plasmids containing these mutations (see Materials and methods). These templates containing either or both Inr1 and Inr2 mutations exhibited reduced efficiencies of MLP transcription *in vitro* and USF[43]-dependent transcription *in vivo* (Du *et al.*, 1993); the same mutations also inhibited TFII-I and USF binding (Roy *et al.*, 1991; Du *et al.*, 1993). As shown in Figure 9, none of these mutations altered the downstream interactions of f:TFIID (lanes 4 and 5). As expected, f:TBP gave only restricted TATA footprints irrespective of Inr mutations (lanes 1−3). We conclude that

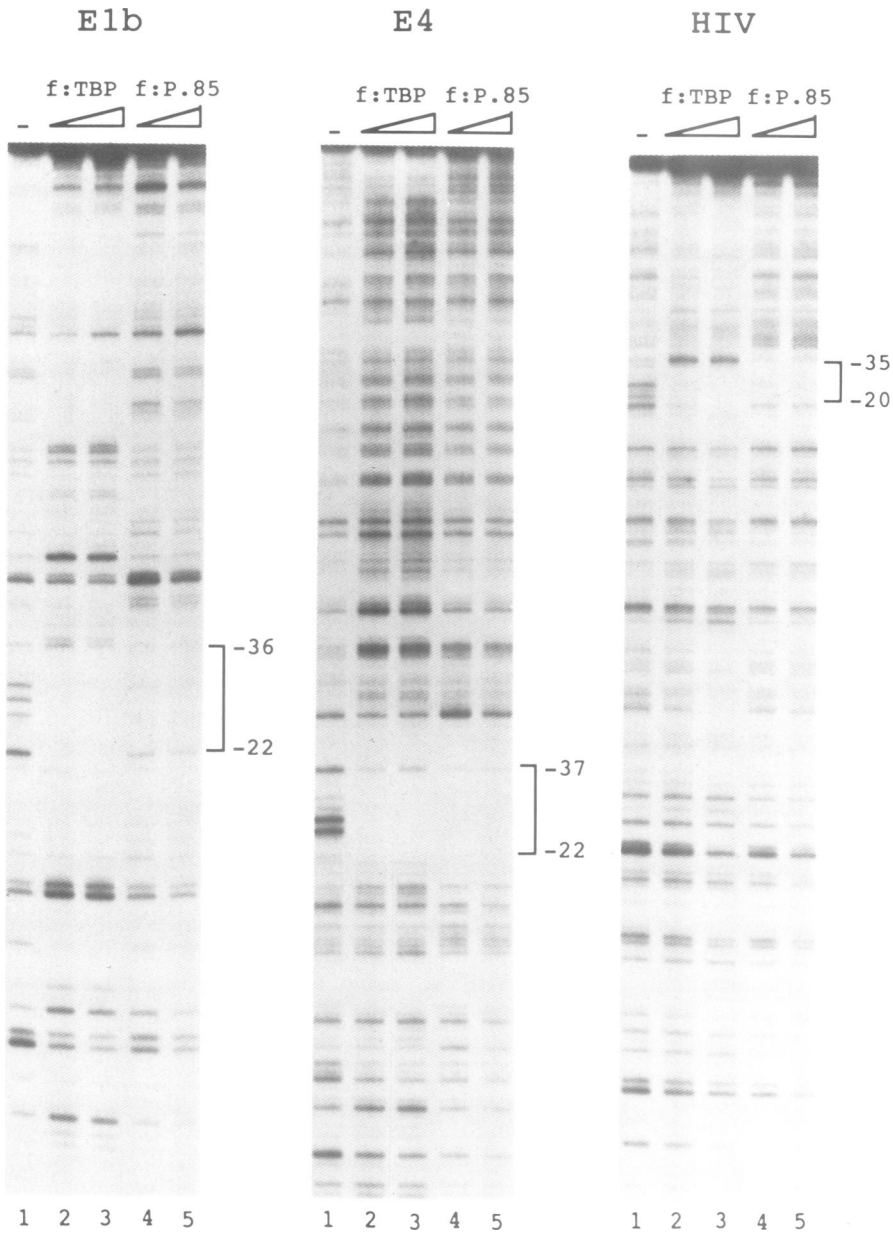

Fig. 10. Restricted footprints of f:TFIID on the E1b, E4 and HIV promoters. DNA templates containing the E1b, E4 and HIV promoters were generated by PCR as described in Materials and methods. The amounts of protein used were the same as indicated in Figure 9. The protected TATA region in each promoter is bracketed.

at least some promoter sequences important for optimal promoter function and for TFII-I binding are not important for downstream TFIID interactions.

Interactions of f:TFIID with E1b, E4 and HIV promoters are restricted to the TATA region

To rule out the possibility that downstream interactions might be caused by contaminants in f:TFIID, we performed DNase I footprinting on two promoters (adenovirus E1b and E4) containing no Inr-like elements and on another promoter (HIV) with typical Inr-like sequences (Roy *et al.*, 1991; Du *et al.*, 1993). Surprisingly, interactions of f:TFIID on these promoters were all restricted to the TATA regions, covering only 16 nucleotides or so, and were similar to those observed with bacterially expressed TBP (Figure 10). On the E1b promoter, TBP also binds to the upstream A/T-rich sequences present in the vector. These results (Figure 10) as well as the Inr mutational studies on the MLP templates (Figure 9) suggest that f:TFIID interactions on various TATA-containing promoters transcribed by RNA polymerase II are determined primarily by sequences at and surrounding individual TATA boxes, or the sequence context between the TATA and initiator elements, but not by the Inr-like sequences themselves. The previous demonstration of persistent downstream interactions on MLP G-less templates with 3′ deletions extending to +10 further supports this viewpoint (Sawadogo and Roeder, 1985).

Discussion

The observations that TBP is required for transcription by RNA polymerases I, II and III and that specific arrays of associated polypeptides (TAFs) are important for both RNA polymerase/promoter specificity and the function of gene-

specific activators on corresponding target promoters necessitates a further definition of the structure and function of the responsible TAFs, including possible relationships to other essential factors or cofactors. To that end, we have used an epitope-tagging method to isolate and characterize distinct TBP-containing complexes for transcription by RNA polymerases II and III. Our results not only complement and extend previous studies using related approaches (Zhou *et al.*, 1992) but also provide an efficient method for isolating relatively pure but structurally complex preparations of natural TBP–TAF complexes.

Unique compositions of TBP–TAF complexes utilized by different RNA polymerases

Compositions of TAFs in various TBP complexes involved in transcription by three RNA polymerases are very different. The pol I transcription factor SL1 contains three TAFs with molecular weights around 110, 63 and 48 kDa (Comai *et al.*, 1992), which seem to be distinct from those TAFs identified in complexes utilized by RNA polymerases II and III (summarized in Figure 4). The TAFs identified in f:TFIID and f:TFIIIB all appear different on the basis of estimated sizes, except that the relationship between the 95 kDa polypeptide of f:TFIID and the 96 kDa polypeptide of f:TFIIIB is not yet clear. Therefore, it seems likely that no common subunit will be shared by these three TBP complexes. In view of the functions exhibited by SL1, TFIID and TFIIIB, it seems that combinations of different TAFs may confer the specific interactions between promoter-specific regulators, RNA polymerases and their accessory factors.

The polypeptide compositions of the f:TFIIIB and f:TFIID preparations described here are respectively more complex than those previously reported for affinity purified TFIIIB (Lobo *et al.*, 1992; Taggart *et al.*, 1992) and for epitope-tagged/affinity purified TFIID (Pugh and Tjian, 1991; Tanese *et al.*, 1991; Takada *et al.*, 1992; Zhou *et al.*, 1992). At least 13 TAFs copurified with TBP from f:TFIID and five major TAFs (230, 135, 95, 80 and 55 kDa) seem to be present in relatively stoichiometric ratios as detected by silver staining (Figures 4 and 5; Zhou *et al.*, 1992) and Coomassie Blue staining (data not shown). However, p135 and p80 showed poor staining by Ponceau S and amido black after transfer on to PVDF membranes, in contrast to p230, p95 and p55, which exhibited more uniform staining patterns (data not shown). These somewhat distorted variances, depending upon the staining reagents used, make evaluation of relative protein abundances difficult. Nevertheless, in our purification scheme we did detect additional, specifically associated polypeptides, especially those smaller than TBP. The uniqueness of these smaller TAFs is evident from partial peptide sequence analysis (C.-M.Chiang and R.G.Roeder, unpublished observation), which excluded the possibility that they were derived from the high molecular weight TAFs. Assuming that these new TAFs are relevant to transcription, their identification indicates an additional functional complexity in promoter regulation. It may also be that there exist multiple species of TFIID complexes containing either qualitatively or quantitatively different forms of associated polypeptides, for mediating interactions with various transcription regulators.

Conversely, f:TFIIIB appears to contain a relatively simpler TAF composition which may reflect a more restricted role(s) in interactions with other basal factors involved in transcription by RNA polymerase III. In yeast, TFIIIB was shown to contain only three polypeptides: a 27 kDa TBP, a 70 kDa BRF and a 90 kDa B'' (Kassavetis *et al.*, 1992). In mammalian cells, three components copurified in the TFIIIB fraction: TBP, TAF-172 and a loosely bound fraction (TAF-L) containing an unidentified factor(s) which dissociates from the TBP–TAF-172 complex at 0.6 M KCl (Taggart *et al.*, 1992). Apparently, our f:TFIIIB complex, isolated after 0.3 M KCl washing, retains extra TAFs (96, 87 and 60 kDa TAFs) which might correspond entirely or in part to the undefined TAF-L components. The exact relationship between these factors remains to be determined.

f:P.30 is functionally equivalent to a component of TFIIIB

f:P.30, a TBP-containing complex isolated from the P11 0.30 M KCl fraction, can functionally substitute for one of two TFIIIB subfractions ($TFIIIB_2$) required for synthesis of VA_I RNA and tRNA (two intragenic pol III promoters) in conjunction with a highly purified TFIIIC and RNA polymerase III in our reconstituted pol III-mediated transcription system (Figure 6A). In fact, when a less purified TFIIIC fraction was used, f:P.30 could substitute for both $TFIIIB_1$ and $TFIIIB_2$ in the reconstituted system (Figure 6B), indicating that a functional equivalent of $TFIIIB_1$ is also present in the TFIIIC P11 fraction. Indeed, a protein fraction which shows different chromatographic properties but substitutes functionally for the $TFIIIB_1$ activity has been isolated from the crude TFIIIC P11 fraction (Z.Wang and R.G.Roeder, unpublished data). Therefore, our f:P.30 is functionally equivalent either to the conventional TFIIIB identified with a crude TFIIIC or to one of the two TFIIIB subfractions identified with a highly purified TFIIIC. When two extragenic pol III promoters (7SK and U6) were tested, no transcription from either template was observed (data not shown). This probably reflects the absence of additional promoter-specific factors, such as the proximal sequence element (PSE)-binding protein (Waldschmidt *et al.*, 1991; Murphy *et al.*, 1992; Simmen *et al.*, 1992b), in our reconstituted system. Alternatively, these promoters could require distinct sets of general factors. In footprinting assays, f:TFIIIB does not bind to the TATA box of the major late promoter (Figure 8), indicating that specific TAFs in this complex might prevent TBP from binding to the TATA boxes of promoters transcribed by RNA polymerase II and possibly the TATA boxes of promoters (7SK, U6) transcribed by RNA polymerase III. This result is consistent with the recent report that TAF-172 (also isolated from P.30), by association with TBP, inhibits transcription by RNA polymerase II (Taggart *et al.*, 1992). The ability of f:TFIIIB (Figure 7) and, similarly, B-TFIID (Timmers and Sharp, 1991; Timmers *et al.*, 1992) to support basal but not activator-dependent transcription assays by RNA polymerase II might be due to some free forms of TBP dissociating from the complexes during the assays.

High levels of activator-dependent transcription by RNA polymerase II requires both native TFIID and the USA cofactor fraction

USA is a cofactor fraction previously shown to be required, in conjunction with partially purified native TFIID, for mediating high levels of activator-dependent transcription

in a more purified reconstituted system (Meisterernst *et al.*, 1991). It contains both a negative cofactor (NC1) that alone represses basal promoter activity and positive cofactors (PC1 and PC2) that potentiate activator function in combination with the negative cofactor. Both NC1 and the functionally similar NC2 compete with TFIIA for interactions with TBP and can prevent the assembly of functional preinitiation complexes on promoters (Meisterernst *et al.*, 1991; Meisterernst and Roeder, 1991). A similar negative cofactor, Dr1 (19 kDa), that also interacts with TBP and inhibits both basal and activated levels of transcription has been purified and cloned from HeLa cells (Inostroza *et al.*, 1992). Given that the partially purified TFIID fractions used in previous studies of activator function were relatively crude, and might have contained various non-specific repressors or anti-repressors, it was possible that the USA cofactor fraction might have been uniquely required in the presence of such components. In addition, while evidently quite pure, the structural complexity of the f:TFIID was such that one might have anticipated that it would contain coactivators sufficient for the function of various activators. In light of these arguments, and the results of other studies which failed to show a USA requirement, it is striking and significant that with the highly purified f:TFIID we again have demonstrated that USA is indispensable for high levels of activator-dependent transcription (Figure 7). This result clearly indicates that cofactors present in the USA fraction, but distinct from the TAFs in TFIID, are essential for high (and more physiological) levels of activated transcription. In other systems using either a highly purified epitope-tagged TFIID (Zhou *et al.*, 1992) or a reconstituted TFIID fraction (Tanese *et al.*, 1991), a possible requirement for USA components may have been obscured by their presence in other less purified factor preparations or by the analysis of activator function under conditions where only modest (a few fold) activation levels were evident. Thus the present use of a more defined reconstituted system makes it clear that cofactors other than those present in the now structurally well defined TFIID are still required for activator function. Although there has been a tendency to view the TFIID subunits primarily as coactivators, it also may be that TFIID, like USA, contains a combination of negatively and positively acting cofactors which cumulatively set the stage for restricted basal and enhanced activator-dependent transcription.

The extended downstream interactions of f:TFIID on the major late promoter are not conferred by initiator-like sequences and may be induced as part of the activation mechanism on other promoters

It has been known for some time that partially purified TFIID preparations have the inherent property of showing interactions over a broad region of certain promoters (Parker and Topol, 1984; Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988; Nakatani *et al.*, 1990) and this has now been verified to be an intrinsic property of the highly purified f:TFIID (Figure 8; Zhou *et al.*, 1992). These interactions are of interest from a functional standpoint because they correlate with core promoter strength in *in vitro* transcription assays. Thus the extremely active adenovirus MLP shows extended footprints with f:TFIID (Figure 8) while the weak (*in vitro*) E1b, E4 and HIV core promoters show footprints restricted to the TATA regions (Figure 10). Most interestingly, however, upstream activators have been shown to induce downstream TFIID interactions on the E4 promoter (Horikoshi *et al.*, 1988a,b), coincident with an increased capability for promoter recognition by other general factors and concomitant assembly of a functional preinitiation complex. These observations argue for the general functional relevance of downstream interactions, and suggest that they may be induced either by sequences intrinsic to certain (strong) core promoters or by regulatory factors on other promoters.

The observations that Inr-like sequences contribute to the activity of the MLP (Du *et al.*, 1993) suggested that they may also be important for downstream TFIID interactions. However, mutations which altered the function of these sequences had no effect on the TFIID downstream interactions, consistent with the demonstration of a distinct Inr-binding factor (TFII-I; Roy *et al.*, 1991) that is not present in f:TFIID (data not shown) or in anti-TBP antibody-purified TFIID (Takada *et al.*, 1992). In support of this, downstream TFIID interactions were not evident on the HIV promoter containing Inr elements related to those in the MLP. These data, plus the fact that similar extended footprints were observed on MLP templates with different downstream sequences after +10 (Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988), indicate that the sequences important for downstream protection on the MLP are located between the TATA and Inr elements, but are not the Inr-like elements themselves. The sequence contexts unique to individual promoters in this region might affect the conformation of TFIID and/or the DNA which in turn gives the characteristic footprints and functional properties of these particular promoters. Alternatively, the induction of such interactions by gene-specific activators could be a point of control in other promoters.

In summary, we have identified two TBP complexes and associated TAFs that are involved in transcription by RNA polymerases II and III by functional and physical binding studies. The complexity of the TAF compositions complements and extends the spectrum of the previously identified TAFs. Moreover, the requirement of the USA cofactor fraction for mediating high levels of activator-dependent transcription was again revealed with this highly purified native f:TFIID. We also demonstrated the function of the highly purified f:TFIIIB in pol III-mediated transcription using both TBP-dependent and TFIIIB-dependent transcription systems. These functional and physical characterization studies of TBP complexes further emphasize the importance of TAFs in determining promoter/polymerase specificity in mammalian transcription.

Materials and methods

Plasmid constructions

Plasmids pFLAG(S)-7 and pFLAG(AS)-7 containing the FLAG epitope in different orientations were constructed as follows. Double-stranded oligonucleotides encoding the FLAG epitope (Hopp *et al.*, 1988; Prickett *et al.*, 1989), an enterokinase cutting site and a heart muscle kinase phosphorylation site (Blanar and Rutter, 1992) were prepared by synthesizing two oligonucleotides (5′-CATGGACTACAAAGACGATGACGATAAA-GCAAGAAGAGCATCTGTGCA-3′ and 5′-TATGCACAGATGCTCTT-CTTGCTTTATCGTCATCGTCTTTGTAGTC-3′) separately, mixing and heating at 90°C for 5 min, and then slowly cooling to room temperature. The resulting hybrid was cloned into the *Nco*I and *Nde*I sites of N:HApGEM-7 (5′Sma) and N:HApGEM-7 (3′Sma) to generate pFLAG(S)-7 (Chiang and Roeder, 1993) and pFLAG(AS)-7, respectively. N:HApGEM-7 (5′Sma) and N:HApGEM-7 (3′Sma) were created by inserting a double-stranded

DNA fragment (the sense strand, 5′-GGGAGATCTGTGAACCACCATGGGATACCCTTATGCTGTTCCTGATTATGCCTCCCTGGGACATATG-3′) which contains a consensus Kozak sequence (Kozak, 1986), the influenza HA epitope sequence (Wilson *et al.*, 1984), and additional *Bgl*II, *Nco*I and *Nde*I sites into the *Sma*I site of pGEM-7Zf(+) (Promega) at opposite orientations (e.g. 5′Sma means that the original *Sma*I site of the vector is at the 5′ end of the insert). The *Nde*I and *Bam*HI fragment of the human TBP cDNA was cloned from pTBP-7 (see below) into pFLAG(S)-7 at the same restriction enzyme cutting sites to create pF:TBP(S)-7 (Chiang and Roeder, 1993). Similarly, the *Nde*I and *Eco*RI fragment of the TBP cDNA was also cloned from pTBP-7 into pFLAG(AS)-7 to create pF:TBP(AS)-7. pTBP-7 is a reconstructed human TBP cDNA clone extending from nucleotide (nt) 1 to nt 1710 (Hoffmann *et al.*, 1990) and including the entire protein-coding and downstream polyadenylation sequences with the *Nde*I site created at the initiation codon at nt 99. The backbone of pTBP-7 is from pGEM-7Zf(+) with additional cloning sites from pBluescript SK− (Stratagene). The multiple cloning sites flanking the TBP cDNA in pTBP-7 are as follows: T7 promoter-*Apa*I-*Aat*II-*Sph*I-*Xba*I-*Xho*I-*Hin*cII/*Acc*I/*Sal*I-*Cla*I-*Hin*dIII-*Eco*RV-*Eco*RI-human TBP cDNA-*Eco*RI-*Pst*I-*Sma*I-*Bam*HI-*Sac*I-*Bst*XI-*Nsi*I-SP6 promoter. The FLAG-TBP coding sequence was then cloned from pF:TBP(S)-7 into pET-11d (Novagen) at the *Nco*I and *Bam*HI sites to generate pF:TBP-11d for protein expression in bacterial BL21(DE3)pLysS cells as previously described (Studier *et al.*, 1990; Chiang and Roeder, 1993). The HF:TBP expression plasmid containing HA/FLAG dual epitopes was similarly cloned as described above except using two different oligonucleotides, 5′-TATCGACTACAAAGACGATGACGATAAAGCAAGAAGAGCATCTGTGCA-3′ and 5′-TATGCACAGATGCTCTTCTTGCTTTATCGTCATCGTCTTTGTAGTCGA-3′, and cloned into the *Nde*I site of N:HApGEM-7 (3′Sma) to generate pHA/FLAG(AS)-7. The *Nde*I and *Sac*I (blunt-ended by the Klenow enzyme) fragment of the TBP cDNA was cloned from pTBP-7 into the *Nde*I and *Eco*RI sites (also blunt-ended by the Klenow enzyme) of pHA/FLAG(AS)-7 to generate pHF:TBP(AS)-7. The *Nco*I and *Bam*HI fragment of the HA/FLAG-TBP insert from pHF:TBP(AS)-7 was then cloned into pET-11d to generate pHF:TBP-11d for bacterial protein expression. pTBP-11a and p6His:TBP-11d expressing the untagged and 6-histidine-tagged TBP were constructed by cloning the *Nde*I and *Bam*HI fragment of the TBP cDNA from pTBP-7 into pET-11a and pET-15b (Novagen, originally called 6HisT-pET11d, Hoffmann and Roeder, 1991), respectively.

pMT2-F:TBP and pMT2-HF:TBP were constructed by cloning the FLAG-TBP and HA/FLAG-TBP inserts (between *Bam*HI and *Eco*RI sites) from pF:TBP(AS)-7 and pHF:TBP(AS)-7, respectively, into the *Eco*RI site of pMT2 (Kaufman *et al.*, 1989) using blunt-end ligation. The *Eco*RI fragment of the TBP cDNA from pTBP-7 was also cloned into the *Eco*RI site of pMT2 to generate pMT2-TBP. These plasmids were used in transient transfection assays in COS-7 cells for protein localization studies.

pBn-F:TBP and pBn-HF:TBP were constructed by transferring the FLAG-TBP and HA/FLAG-TBP inserts (between *Bam*HI and *Eco*RI sites) from pF:TBP(AS)-7 and pHF:TBP(AS)-7, respectively, into pBabe Neo, a retroviral vector conferring G418-resistant phenotype in transfected cells (Morgenstern and Land, 1990).

The major late promoter (MLP) plasmid, pML4, used for DNase I footprinting was created by inserting a PCR amplification fragment between −91 (*Xba*I) and +85 (*Bam*HI) into the *Xba*I and *Bam*HI sites of pBS+ (Stratagene) as previously described (Horikoshi *et al.*, 1992). The plasmids, MLI, MLI_1R, MLI_2 and MLI_1R-I_2, containing, individually, MLP wild-type, initiator 1, initiator 2, and dual initiator 1 and 2 mutations between −45 and +65 in pCAT-Basic (Promega) have been described before (Du *et al.*, 1993). Plasmids E1b-CAT, E4-CAT (Lillie and Green, 1989) and NF_1HCAT (Du *et al.*, 1993) contain the E1b, E4 and HIV promoters, respectively, in pSP72 (Promega).

pVA_I containing the adenovirus VA_I promoter was constructed by ligating the 231 bp *Xba*I−*Bal*I restriction fragment from pA2wt (Hoeffler and Roeder, 1985) into an *Xba*I/*Sma*I-cut pUC12 vector (constructed by M.W.Van Dyke). The plasmid, pG_5HMC_2AT, containing five GAL4 binding sites preceding the HIV TATA box and MLP initiator element in front of a G-less cassette, was obtained from R.Bernstein. $pXltmet_1$ (Engelke *et al.*, 1980) and pMLΔ53 (Sawadogo and Roeder, 1985; Meisterernst *et al.*, 1991) contain the tRNA and core MLP, respectively.

Indirect immunofluorescence

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transient transfections were conducted in chambered slides using 10 μg of pMT2-TBP, pMT2-F:TBP or pMT2-HF:TBP DNA as previously described (Chiang *et al.*, 1991). 48 h later, cells were incubated with 5 μg/ml of anti-FLAG M2 monoclonal antibody (IBI/Kodak) or anti-E1A M2 monoclonal antibody (Harlow *et al.*, 1985) in 5% goat serum using the procedure described by Labow *et al.* (1990).

Establishment of HeLa cell lines constitutively expressing f:TBP by retrovirus-mediated gene transfer

The retroviral packaging cell line, Ψ_{CRIP} (Danos and Mulligan, 1988), was grown in DMEM supplemented with 10% calf serum. It was used to generate the amphotropic viral particles for infection of HeLa cells. Briefly, 20 μg of pBn-F:TBP, pBn-HF:TBP or pBabe Neo DNA were individually transfected into Ψ_{CRIP} cells at 50% confluency in 100 mm plates using the calcium phosphate−DNA coprecipitation method, as described by Graham and van der Eb (1973) and modified by Parker and Stark (1979). Supernatant containing viral particles was harvested 18 h after the glycerol shock and filtered through a 0.45 μm Nalgene filter (Cepko *et al.*, 1984). 1 ml of the viral supernatant was mixed with 1 ml of DMEM/10% FBS and polybrene (final concentration 8 μg/ml) and used to infect HeLa cells which were grown in DMEM/10% FBS at 50% confluency in 100 mm plates. After incubation at 37°C for 2.5 h, an additional 8 ml of DMEM/10% FBS was added to each plate. Cells were incubated at 37°C for another 1.0−1.5 days before being split 1:6 for G418 selection (1 mg/ml; the active component is ~0.5 mg/ml). Medium was changed every 3 or 4 days. Individual G418-resistant colonies, normally seen after a 2-week selection, were expanded into cell lines and then characterized by Western blotting using the anti-FLAG M2 monoclonal antibody. Two cell lines (3-9 and 3-10) expressing FLAG-TBP and one cell line (4-5) expressing HA/FLAG-TBP were further adapted to large spinner culture maintained in Joklik's medium supplemented with 5% calf serum. Cells were finally switched to DMEM-phosphate medium containing 5% calf serum and used for preparation of nuclear extracts.

Western blotting

Subconfluent cells in 60 mm plates were washed once in 1 × phosphate buffered saline (PBS), drained well, and lysed in 300 μl of 1 × Laemmli's buffer. 10 μl samples were loaded onto a 10% polyacrylamide−SDS gel. Western blotting was performed as described by Harlow and Lane (1988) using 3 μg/ml of the anti-FLAG M2 monoclonal antibody. Signals were detected using the ECL system (Amersham) according to the manufacturer's instructions.

Immunopurification of f:TBP complexes by FLAG-peptide elution

Nuclear extracts were prepared as previously described (Dignam *et al.*, 1983). For a preliminary test, 20 ml of nuclear extract was fractionated on a phosphocellulose P11 (Whatman) column containing 15 ml of resin and successively step-eluted with 0.1, 0.3, 0.5 and 0.85 M KCl in BC buffer (20 mM Tris−HCl, pH 7.9 at 4°C, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM dithiothreitol). Fractions collected from 0.5 and 0.85 M KCl elutions were further dialyzed against BC100 (BC buffer plus 100 mM KCl) at 4°C for 5 h. 200 μl of each P11 fraction was incubated with 10 μl of the anti-FLAG M2-agarose (IBI/Kodak) at 4°C for 6 h by rotation. After washing five times in BC300 (BC buffer including 300 mM KCl) plus 0.1% Nonidet P-40, the bound proteins were eluted from the M2-agarose by incubation at 4°C for 20 min with 20 μl of BC300−0.1% Nonidet P-40 plus 0.2 mg/ml of the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, synthesized by the Protein Sequencing Facility of The Rockefeller University). 5 μl aliquots of eluted samples were mixed with equal volumes of 2 × Laemmli's buffer and loaded onto a 10% polyacrylamide−SDS gel. Proteins were visualized by silver staining using the Rapid-Ag-Stain kit (ICN) according to the manufacturer's instructions.

For transcription and physical binding studies, 14 ml of P11 0.3 M and 0.85 M fractions from 3-10 cells were incubated with 500 μl of the M2 agarose at 4°C for 6 h by rotation. After washing five times in BC300−0.1% Nonidet P-40, the M2-agarose with bound proteins was transferred to a microcentrifuge spin column (Invitrogen). Residual liquid in the resin was removed by a quick spin. The protein complexes were eluted from the M2-agarose resin by incubation with 400 μl of BC300−0.1% Nonidet P-40 plus 0.2 mg/ml of the FLAG peptide at 4°C for 20 min. The eluate was collected by spinning at 4°C for 10 s and designated as the first eluted fraction. Peptide elutions were repeated another three times as described above. Purification of the bacterially expressed f:TBP has been described (Chiang and Roeder, 1993).

Fractionations of TFIIIB, TFIIIC and RNA polymerase III

To purify TFIIIB, 100 ml of HeLa S100 was loaded onto a P11 phosphocellulose column in BC100, then step-eluted with BC350 and BC600 as previously described (Segall *et al.*, 1980). The P11 0.35 M KCl fraction of TFIIIB (15 ml/22 mg) was applied to an FPLC HR5/5 Mono Q column

(Pharmacia) in Buffer A (20 mM HEPES–NaOH, pH 7.9, 10% glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 3 mM DTT, 0.5 mM PMSF) containing 100 mM KCl, and eluted with a 25 ml linear gradient from 100 to 500 mM KCl in Buffer A. $TFIIIB_1$ was eluted at 210 mM KCl, while $TFIIIB_2$ was eluted at 280 mM KCl. TFIIIC was partially purified according to the published procedure (Kovelman and Roeder, 1992) except that DNA–cellulose and oligonucleotide affinity columns were omitted. Briefly, 170 ml of HeLa nuclear extract was loaded on a heparin–Sepharose 4B (Pharmacia) column, and the TFIIIC activity was eluted with 0.6 M KCl in BC buffer. The TFIIIC fraction was further purified through P11 and HPLC SP-5PW (Bio-Rad) columns. TFIIIC purified after the HPLC SP-5PW column did not contain TBP and was used for transcription assays. RNA polymerase III was purified from HeLa S100 as described (Kovelman and Roeder, 1990). One unit of activity represents incorporation of 1 pmol of UMP into RNA in 20 min using poly[d(A-T)] (Boehringer Mannheim Biochemicals) as template (Sklar and Roeder, 1976).

In vitro transcription assays by RNA polymerase III

A 25 μl reaction mixture contained 0.5 μg of DNA template, 10 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 70 mM KCl, 0.5 mM DTT, 25 μM of [α-^{32}P]GTP, 600 μM each of ATP, CTP and UTP, and protein fractions specified in the figure legend. Reactions were incubated at 30°C for 60 min, and then terminated by adding 25 μl of Stop Solution containing 1% SDS, 100 mM sodium acetate (pH 5.2), 20 mM EDTA and 1 mg/ml of tRNA. RNA was extracted with phenol–chloroform, precipitated and washed with ethanol, and finally dissolved in 10 μl of formamide containing 0.1% each of xylene cyanol FF and bromophenol blue and 10 mM EDTA. Samples were analyzed on an 8% polyacrylamide–7 M urea gel, vacuum-dried and exposed at −70°C using Kodak BB X-ray film.

In vitro transcription assays by RNA polymerase II

General transcription factors and the USA cofactor fraction were purified from HeLa nuclear extracts as previously described (Meisterernst *et al.*, 1991) with the following modifications. Fractions containing TFIIE/F/H from P11 0.5 M KCl elution were applied to a DEAE–cellulose (DE52) column, eluted with 0.3 M KCl, and passed through a double-stranded DNA–cellulose column to remove contaminating TFIID activity. The flowthrough from the double-stranded DNA–cellulose column was applied to an FPLC Mono S column and eluted with 0.3 M KCl after washing the column with BC150. The P11 0.85 M KCl fractions containing TFIID activity were first dialyzed against BC100 and then applied to a DEAE–cellulose column and eluted with BC300. After dialysis against BC100 the 0.3 M KCl fraction was further separated by the FPLC Mono S column and eluted with a linear gradient from 0.1 to 0.7 M KCl in BC buffer. TFIID activity was recovered between 0.3 and 0.4 M KCl. Recombinant histidine-tagged TFIIB, expressed in and purified from *E.coli*, was obtained from S.Malik (Malik *et al.*, 1991). To prepare the USA fraction, the dialysed P11 0.85 M KCl fraction was twice applied to a DE52 column to limit TFIID contamination, and then loaded onto a heparin–Sepharose column from which USA was eluted with 0.5 M KCl. Bacterially expressed GAL4-AH was purified as previously described (Lin *et al.*, 1988). *In vitro* transcription was carried out in a 25 μl reaction mixture containing 100 ng pG_5HMC_2AT, 40 ng pMLΔ53, 1 μl TFIIA (0.5 mg/ml), 10 ng recombinant TFIIB, 1 μl TFIIE/F/H (0.3 mg/ml) and 0.5 μl RNA polymerase II (0.3 mg/ml) in the presence or absence of other tested components using the conditions previously described (Meisterernst *et al.*, 1991) except that no RNase Block II was added. RNA was phenol–chloroform extracted, ethanol precipitated, analyzed directly by 5% polyacrylamide–urea gel electrophoresis and visualized by autoradiography as described above.

DNase I footprinting

DNA templates used for footprinting on the transcribed and non-transcribed strands of the wild-type MLP were prepared by isolating the *Eco*RI–*Hin*dIII fragment from pML4 and end-labeled with ^{32}P by T4 polynucleotide kinase. Specific transcribed and non-transcribed templates were then generated by cleaving the labeled DNA fragments, separately, with either *Pst*I or *Kpn*I. The MLP initiator mutations and corresponding control wild-type templates were produced by using MLI_1R, MLI_2, MLI_1R-I_2 or MLI (Du *et al.*, 1993), separately, as DNA templates for PCR amplification. The upstream primer (5′ 2233–2254 3′, numbering according to pCAT-Basic) includes a *Hin*dIII site and the downstream primer (3′ 2329–2348 5′) is from the CAT gene. The PCR products were end-labeled by T4 polynucleotide kinase and digested with *Hin*dIII. The E1b, E4 and HIV promoter fragments were similarly generated by PCR using E1b-CAT, E4-CAT and NF_1HCAT, respectively, as DNA templates with an upstream primer (5′ 2365–2384 3′, numbering following pSP72) that includes an *Nde*I site and the same downstream CAT primer. The PCR products were end-labeled by T4 polynucleotide kinase and digested with *Nde*I. All DNA templates were finally adjusted to 2 fmol/μl or 20 000 c.p.m./μl in 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA (TE) after phenol–chloroform extraction and ethanol precipitation, and used for binding reactions.

A typical 50 μl binding reaction contained 2 fmol of the ^{32}P-labeled DNA fragment, 0.5 ng of poly[d(G-C)], 5 μg of bovine serum albumin, 10 mM HEPES, pH 7.9, 60 mM KCl, 5 mM DTT, 0.2 mM EDTA, 4 mM $MgCl_2$, 10% glycerol and appropriate amounts of purified proteins. The reaction mixture was incubated at 30°C for 1 h, and then 50 μl of the Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$ + 10 mM $MgCl_2$) was added to each tube at room temperature. 1 μl of DNase I (0.4 ng/μl, diluted in TE before use) was added to each tube and digestion was continued at room temperature for 2 min. The reaction was terminated by adding 100 μl stop solution (0.2 M NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml yeast RNA) and chilled on ice. After phenol–chloroform extraction and ethanol precipitation and washing, the final DNA was resuspended in 5 μl gel loading buffer (86% formamide, 0.02% each of bromophenol blue and xylene cyanol, and 1 × TBE) and loaded on an 8% polyacrylamide–urea sequencing gel. DNA bands were detected by autoradiography after overnight exposure at −70°C with intensifying screens.

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