1 to 4492) and nucleotides 5000 to 6756 from α_{1E} (α_{1E}: 5000 to 6756), with position 1 at the start codon; the α_{1E} fragment was ligated into α_{1C} that had been cut at nucleotide position 4487 with Bcl I, and in the polylinker with Xba I. For α_{1Cdel} (α_{1C}: base pairs 1 to 5196 + TAG), the Bst EII (α_{1C}: 4641) and Xba I (polylinker) region of α_{1C} was replaced with a shorter fragment, including a premature stop codon after the codon for amino acid 1732. For α_{1CE-2} (α_{1C}: 1 to 4492 and α_{1E}: 5000 to 5586 and α_{1C}: 5071 to 6516), the α_{1C} fragment (α_{1C}: 5071 to 6516) was ligated into Sau I (α_{1CE-1}: 5071) and Xba I (polylinker) of α_{1CE-1}. For α_{1CE-3} (α_{1C}: 1 to 4492 and α_{1E}: 5000 to 5187 and α_{1C}: 4681 to 6516), the α_{1C} fragment (α_{1C}: 4681 to 6516) was ligated into Stu I (α_{1CE-1}: 4674) and Xba I (polylinker) of α_{1CE-1}. For α_{1EC-1} (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 6516), the α_{1C} fragment (α_{1C}: 4544 to 6516) was ligated into the Xho I (α_{1E}: 5045) and Sal I (polylinker) sites of α_{1E}. For $\alpha_{1EC-1\Delta4}$ (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 5406 + TAG), the α_{1EC-1} fragment (α_{1EC-1}: 4299 to 5913 + TAG) was ligated into the Bst 1107I (α_{1EC-1}: 4299) and Sal I (polylinker) sites of α_{1EC-1}. For $\alpha_{1EC-1\Delta3}$ (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 5196 + TAG), the α_{1EC-1} fragment (α_{1EC-1}: 4299 to 5703 + TAG) was ligated into the Bst 1107I (α_{1EC-1}: 4299) and Sal I (polylinker) sites of α_{1EC-1}. For $\alpha_{1EC-1\Delta2}$ (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 4980 + TAG), the α_{1EC-1} fragment (α_{1EC-1}: 4299 to 5487 + TAG) was ligated into the Bst 1107I (α_{1EC-1}: 4299) and Sal I (polylinker) sites of α_{1EC-1}. For α_{1EC-3} (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 4947 and α_{1E}: 5464 to 6756), the α_{1E} fragment (α_{1E}: 5464 to 6756) was ligated into Bgl II (α_{1EC-1}: 5369) to Sal I (polylinker) of α_{1EC-1}. For α_{1EC-2} (α_{1E}: 1 to 5050 and α_{1C}: 4544 to 4667 and α_{1E}: 5175 to 6756), the α_{1E} fragment (α_{1E}: 5175 to 6756) was ligated into Bst EII (α_{1EC-1}: 5148) and Sal I (polylinker) of α_{1EC-1}. Portions of chimeric channel constructs derived from PCR were verified in their entirety with the use of the fluorescent dideoxy terminator method of thermocycle sequencing on an automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division 373a).

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40. We thank J. P. Imredy, P. G. Patil, D. L. Brody, B. A. Lewis, and K. W. Yau for discussion and comments; S. Sisodia and M. Schlissel for molecular biological advice; and the DNA Analysis Facility of Johns Hopkins for sequencing. Supported by grants from NIH (D.T.Y. and E.P.-R.) and the Medical Research Council of Canada and the Howard Hughes Medical Institute (T.P.S.); an Established Investigatorship of the American Heart Association (D.T.Y.); a Medical Scientist Training Program award (L.J.); and a National University of Sing- apore fellowship (T.W.S.).

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Coactivator and Promoter-Selective Properties of RNA Polymerase I TAFs

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Human ribosomal RNA synthesis by RNA polymerase I requires the activator UBF and the promoter selectivity factor SL1, which consists of the TATA binding protein (TBP) and three associated subunits, TAF_I110, TAF_I63, and TAF_I48. Here it is shown that both TAF_I110 and TAF_I63 contact the promoter, whereas TAF_I48 serves as a target for interaction with UBF and is required to form a transcriptionally active SL1 complex responsive to UBF in vitro. TAF_I48 also alters the ability of TBP to interact with TATA box elements, and the resulting complex fails to support transcription by RNA polymerase II. Thus, TAF_I48 may function both as a target to mediate UBF activation and as a class-specific promoter selectivity factor.

Enhancement of transcription initiation by sequence-specific DNA binding proteins is a principal mechanism for regulating gene expression in animal cells (*1*). Promoter-selective transcriptional activators bind DNA and interact with specific components of the basal transcriptional apparatus in order to modulate gene expression (*2*). For example, some site-specific enhancer binding proteins directly target subunits of the basal transcription factor TFIID, which consists of TBP and at least eight TBP-associated factors called TAF_{II}s (*3–8*). Transcription of the human 18S and 28S ribosomal RNA genes by RNA polymerase I (RNA Pol I) also requires a TBP-TAF complex called selectivity factor 1 (SL1). All four subunits (TBP, TAF_I48, -63, and -110) are necessary to form an SL1 complex that supports transcription in vitro with purified RNA Pol I and the upstream binding factor, UBF (*9–11*). Consequently, one or more TAFs in the SL1 complex are expected to recognize and bind core promoter DNA, whereas other subunits may be targets for activation by UBF.

To test the activator binding properties of individual TAFs in the SL1 complex, we performed protein-protein binding assays with an affinity resin containing human recombinant UBF (rUBF) (Fig. 1) (*12*). As reported previously, UBF interacts with TBP (*13*). However, TBP alone is unable to support UBF-dependent activation of transcription by RNA Pol I (*11*). We therefore tested each of the TAFs associated with

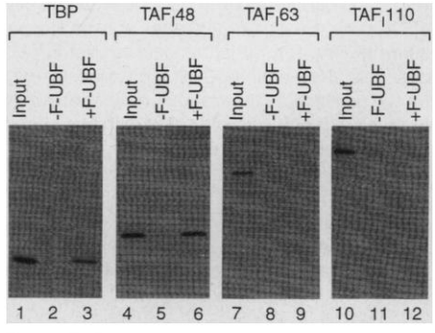

Fig. 1. TAF_I48 subunit of the SL1 complex binds to UBF. Partially purified FLAG epitope–tagged UBF (*12*) was immobilized on protein A–Sepharose beads conjugated with antibodies directed against the FLAG epitope (*10*). This resin (lanes 3, 6, 9, and 12) and resins containing the antibodies but lacking UBF (lanes 2, 5, 8, and 11) were incubated with in vitro ^{35}S-methionine–labeled TBP (lanes 2 and 3), TAF_I48 (lanes 5 and 6), TAF_I63 (lanes 8 and 9), and TAF_I110 (lanes 11 and 12). Bound proteins were resolved by SDS-PAGE and visualized by autoradiography (*10*). Lanes 1, 4, 7, and 10 show 10% of the individual TAFs used in the binding assay.

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SL1 and found that the smallest subunit, TAF_I48, bound efficiently and selectively to the UBF affinity resin (Fig. 1). In contrast, neither TAF_I63 nor TAF_I110 was retained on the UBF resin to a level above background. Thus, TAF_I48 and TBP are potential targets for interaction with UBF.

To test the relevance of the UBF–TAF_I48 interactions, we assembled various TBP-TAF complexes and assessed their ability to mediate UBF-dependent activation (Fig. 2). Transcription reactions supplemented with RNA Pol I and rUBF but no SL1, or reactions containing RNA Pol I and SL1 but no rUBF, produced very low levels of transcription from the human ribosomal promoter (Fig. 2). When either endogenous immunopurified holo SL1 or recombinant SL1 (rSL1) (Fig. 2) was added to these reactions, high levels of transcription were observed. However, when a partial SL1 complex containing TBP-TAF_I63-TAF_I110 but lacking TAF_I48 [rSL1(-48)] (Fig. 2) was added, the level of transcription observed was similar to that found in the absence of endogenous SL1. A triple complex of the three TAF subunits without TBP was also unable to support UBF-dependent transcription (*11*). These results, taken together with the observation that UBF can bind directly to TAF_I48 and TBP, suggest that both TBP and TAF_I48 are required to assemble an active SL1 complex able to mediate UBF-dependent transcriptional activation.

Although human SL1 on its own binds poorly to DNA, it interacts efficiently with the ribosomal RNA (rRNA) promoter in the presence of UBF (*14*). Upon UBF binding to the upstream control element (UCE) of the rRNA promoter, SL1 is recruited to the template DNA and interacts selectively with an upstream region, which produces an extended footprint pattern (*14*) (Fig. 3A). To test the DNA binding properties of a rSL1(−48) complex, we carried out deoxyribonuclease I (DNase I) protection experiments. In the absence of SL1, binding of UBF to the UCE resulted in a defined protected region (−97 to −115), as well as

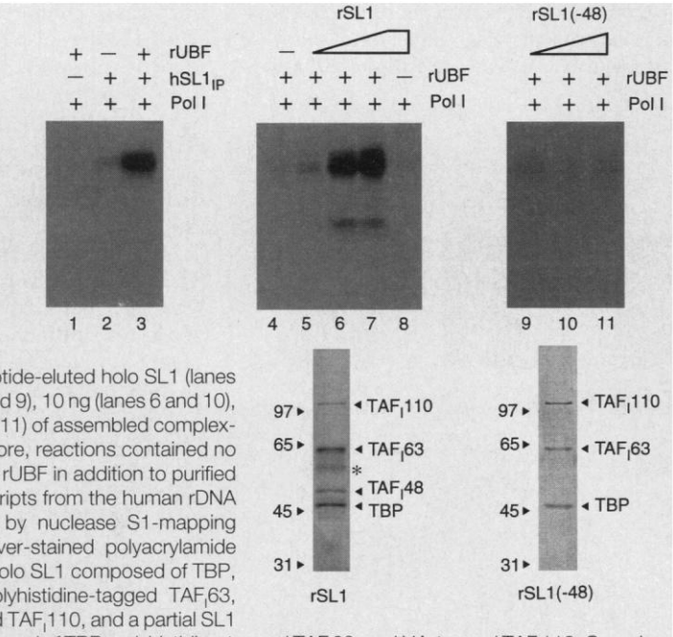

Fig. 2. TAF_I48 is required for transcriptional activation. (**Upper panels**) In vitro transcription reactions used immunopurified and peptide-eluted holo SL1 ($hSL1_{IP}$) derived from HeLa cells (lanes 2 and 3) (*12*), an in vitro assembled quadruple SL1 complex (lanes 5 to 8), or a triple complex lacking TAF_I48 but containing all three SL1 subunits (lanes 9 to 11). Assays contained either ~3 ng of peptide-eluted holo SL1 (lanes 2 and 3) or 5 ng (lanes 5 and 9), 10 ng (lanes 6 and 10), and 20 ng (lanes 7, 8, and 11) of assembled complexes, respectively. Furthermore, reactions contained no (−) or 10 ng (+) of human rUBF in addition to purified RNA Pol I (*12, 26*). Transcripts from the human rDNA promoter were analyzed by nuclease S1-mapping (*11*). (**Lower panels**) Silver-stained polyacrylamide gel of in vitro assembled holo SL1 composed of TBP, FLAG-tagged TAF_I48, polyhistidine-tagged TAF_I63, haemaglutinin (HA)-tagged TAF_I110, and a partial SL1 complex [rSL1(-48)] composed of TBP, polyhistidine-tagged TAF_I63, and HA-tagged TAF_I110. Complexes were built and purified on antibody affinity resin and subsequently eluted with peptides corresponding to the epitopes (*10, 11*). Molecular size standards (in kilodaltons) are indicated on the left. Proteins and traces of immunoglobulin heavy chain (asterisk) are indicated on the right.

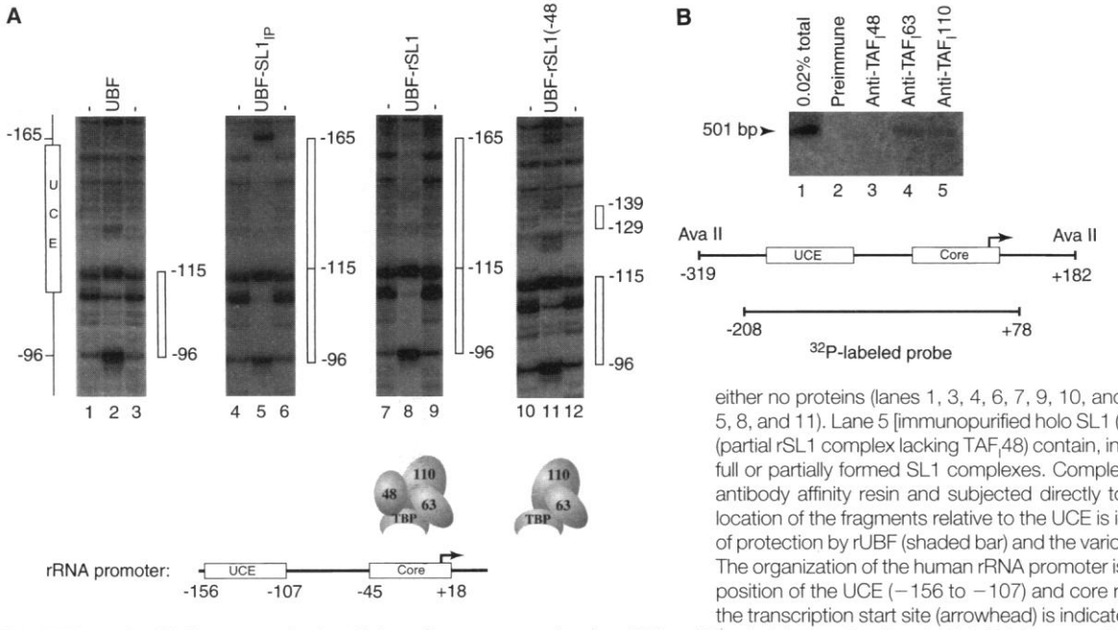

Fig. 3. DNA binding properties of TAF_Is. (**A**) A partial rSL1 lacking TAF_I48 interacts with the human rRNA promoter. DNase I footprint analysis of rUBF, endogenous immunopurified holo SL1 ($SL1_{IP}$), assembled rSL1, and an assembled rSL1(−48) complex on the human rRNA promoter. A DNA fragment containing the ribosomal core promoter sequence [−500 to +78; (*14*)] was incubated with either no proteins (lanes 1, 3, 4, 6, 7, 9, 10, and 12) or purified rUBF (lanes 2, 5, 8, and 11). Lane 5 [immunopurified holo SL1 (*12*)], lane 8 (rSL1), and lane 11 (partial rSL1 complex lacking TAF_I48) contain, in addition to UBF, the indicated full or partially formed SL1 complexes. Complexes were built and purified on antibody affinity resin and subjected directly to footprint reactions (*27*). The location of the fragments relative to the UCE is indicated in addition to regions of protection by rUBF (shaded bar) and the various SL1 complexes (white bar). The organization of the human rRNA promoter is shown schematically and the position of the UCE (−156 to −107) and core region (−45 to +18) relative to the transcription start site (arrowhead) is indicated (*14*). (**B**) Distribution of RNA Pol I TAFs on the rDNA promoter in vivo. HeLa cells, grown to a density of 7.5×10^5 cells/ml, were irradiated with UV light to covalently cross-link protein to DNA. These protein-DNA complexes were purified as described (*28*), restriction digested with endonuclease Ava II, and fragments immunoprecipitated with antibodies directed against each TAF_I (lanes 3 to 5) or preimmune serum (lane 2). The immunoprecipitated DNA fragment was visualized by DNA blot hybridization with a ^{32}P-labeled DNA probe that spans the rDNA promoter. A percentage of the total amount of DNA that was present in the immunoprecipitation reaction is included (0.02%, lane 1). Each immunoprecipitation contains ~150 μg of DNA.

an enhanced cleavage site at position −96 (Fig. 3A). However, when endogenous holo SL1 was present in the reaction with UBF, protected regions covered the entire UCE and extended to position −165 (Fig. 3A). A similar extended footprint was seen with rSL1 (Fig. 3A). In contrast, a rSL1 complex lacking TAF_I48 produced a smaller and weaker pattern of protection (−129 to −139; Fig. 3A). A similar restricted weak footprint was also seen with UBF and a dimeric complex consisting of TBP and TAF_I63 (*12*), suggesting that TAF_I63 can bind directly to promoter DNA sequences. Thus, although complexes lacking TAF_I48 retain some ability to interact with the rRNA promoter, they do so in an altered fashion and are not active transcriptionally.

To determine whether TAF_Is other than TAF_I63 contact promoter DNA directly in the context of a native SL1 complex, we performed in vivo ultraviolet (UV) cross-linking experiments (Fig. 3B). HeLa cells were exposed to UV light, and the resulting protein-DNA adducts were immunoprecipitated with antibodies to TAF_I. After immunoprecipitation with antibodies specific for TAF_I63 (anti-TAF_I63) and TAF_I110 (anti-TAF_I110), a DNA fragment of the expected size was observed. In contrast, no promoter fragments were immunoprecipitated by control serum or anti-TAF_I48. These experiments support our in vitro finding that TAF_I63 can bind directly to promoter DNA. In addition, TAF_I110 may also be a DNA binding component within the SL1 complex. These results, together with our DNase I footprint experiments with rSL1(−48), suggest that TAF_I110 may be responsible for the fully extended footprint pattern of holo SL1 and that this extended DNA binding may be dependent on TAF_I48 as well as UBF.

RNA Pol I core promoter elements differ from RNA Pol II core promoters (*15*, *16*), and therefore TAFs may control the template recognition properties of TBP. We wanted to identify TAFs in SL1 that could modify the DNA binding properties of TBP and target SL1 to the RNA Pol I promoter. Whereas purified TBP alone produced a clear protected region centered around the AdML TATA box, TBP in a dimeric complex with TAF_I48 was unable to bind and protect the TATA box (Fig. 4A). In contrast, TBP in a complex with TAF_I63 or TAF_I110 can interact with TATA sequences (*12*). These results suggest that binding of TAF_I48 to TBP alters the ability of TBP to either recognize or bind to cognate TATA box sites.

On the basis of these binding studies, one might hypothesize that class-specific TAFs can alter the promoter specificity of TBP-TAF complexes. However, it was possible that even if TBP cannot bind to its recognition site on the template, it nevertheless retains its ability to direct transcription by RNA Pol II (*16*, *17*). Therefore, we assessed the ability of TBP-TAF_I48 complexes to mediate transcription by RNA Pol II from the AdML promoter in vitro. First, we compared the ability of TBP with that of a dimeric TBP-TAF_I48 complex (Fig. 4A) to direct basal levels of transcription from the AdML promoter in the presence of RNA Pol II and all of the requisite accessory factors of its basal machinery (Fig. 4B). When in vitro reactions were conducted without added TBP, no detectable basal transcription by RNA Pol II was observed. When purified TBP (Fig. 4A) was added to the reaction, accurate initiation of transcription was observed. In contrast, if an equimolar amount of the TBP-TAF_I48 dimeric complex was added to the reaction, transcription was not detected. However, the inability of a TBP-TAF_I48 dimeric complex to direct transcription was restored by addition of an excess of free TBP to reactions containing these dimeric complexes (Fig. 4B). As a

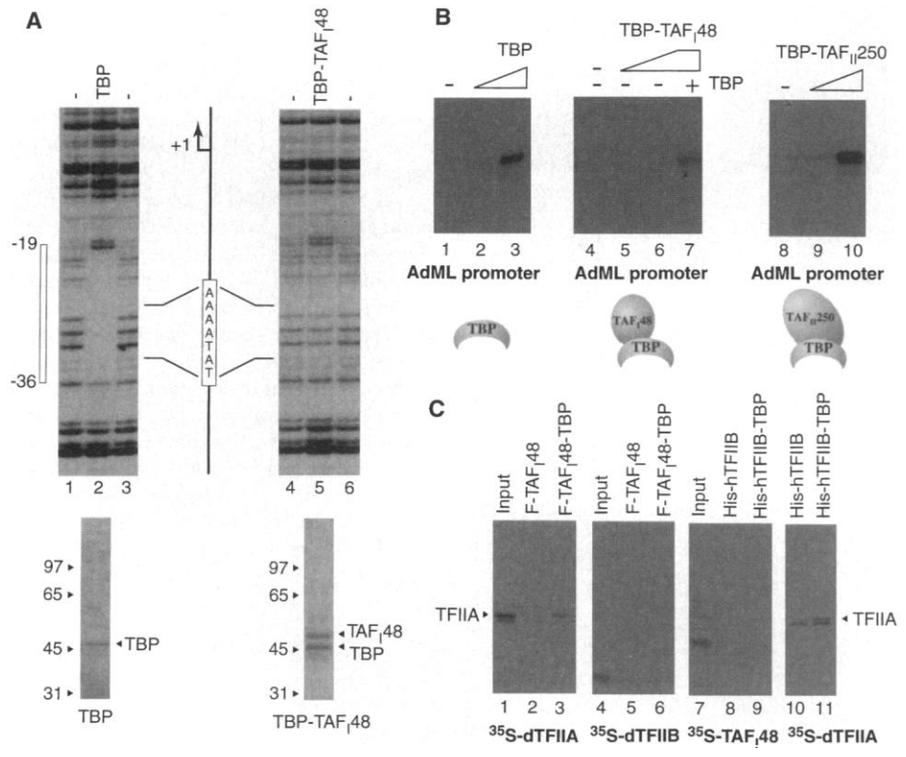

Fig. 4. TAF_I48 modulates the properties of TBP. (**A**) (**Upper panels**) Binding of TAF_I48 to TBP prevents TATA-box recognition. DNase I footprint analysis of TBP and TBP-TAF_I48 dimeric complex on the AdML promoter. A DNA fragment containing the AdML core promoter sequence (−116 to +61) was incubated with either no protein (lanes 1, 3, 4, and 6), 15 ng of purified TBP (lane 2), or an equivalent amount of TBP in a dimeric complex with TAF_I48 (lane 5) before partial digestion by DNase I (*20*). The location of the fragments relative to the transcription start site (arrowhead) is indicated. The sequence of the TATA box (−25 to −30) is shown in addition to the region of protection by TBP (−19 to −36). (**Lower panels**) Silver-stained polyacrylamide gel of purified human TBP (*11*) and in vitro assembled dimeric TBP-FLAG–tagged TAF_I48 (*10*) complex. Molecular size standards (in kilodaltons) are indicated on the left. Proteins are indicated on the right. (**B**) TBP-TAF_I48 complexes are unable to promote RNA Pol II transcription. TBP, TBP-TAF_I48 complex, and TBP-$TAF_{II}250$ complex were tested for their ability to support basal levels of transcription from the AdML promoter by RNA Pol II in a human in vitro transcription system (*5*). Each reaction contains, in addition to RNA Pol II, all of the requisite accessory factors (TFIIA, -IIB, -IIE, -IIF, and -IIH) essential for transcription by RNA Pol II. Reactions were performed either in the absence of the indicated proteins (lanes 1, 4, and 8) or in the presence of 3 ng and 20 ng of TBP (lanes 2 and 3) or with approximately equimolar amounts of TBP in complex with TAF_I48 (lanes 5 to 7) or $TAF_{II}250$ (lanes 9 and 10). Reactions in lanes 4 to 7 contain in addition no (−) or 20 ng (+) of TBP. Transcripts were analyzed by primer extension (*5*). (**C**) TAF_I48 excludes TFIIB but not TFIIA from binding to TBP. FLAG epitope–tagged TAF_I48 was immobilized on protein A–Sepharose beads containing anti-FLAG (*10*). Resin (lanes 2 and 5) and resins containing a TAF_I48-TBP dimeric complex (lanes 3 and 6) were incubated with in vitro ^{35}S-methionine–labeled *Drosophila* TFIIA (lanes 2 and 3) (*23*) or *Drosophila* TFIIB (lanes 5 and 6). Resin containing polyhistidine-tagged human TFIIB (*25*) (lanes 8 and 10) or a human TFIIB-TBP complex (lanes 9 and 11) (*12*) were incubated with in vitro ^{35}S-methionine–labeled TAF_I48 (lanes 8 and 9) or dTFIIA (lanes 10 and 11). Proteins were resolved by SDS-PAGE and analyzed by autoradiography (*10*). Lanes 1 (dTFIIA), 4 (dTFIIB), and 7 (TAF_I48 show 10% of the individual ^{35}S-methionine–labeled proteins used in the binding assays. The correct size of large dTFIIA subunit is indicated.

control, we assembled a partial TFIID complex containing TBP and $TAF_{II}250$ (*5*, *12*). This dimeric complex, unlike the TBP-TAF_I48 dimer, fully supported basal transcription by RNA Pol II (Fig. 4B). Consistent with these results is the inability of SL1 to substitute for TFIID on RNA Pol II promoters and vice versa (*12*). Taken together, these results establish that TAF_I48 can alter the DNA binding properties of TBP so that it can no longer recognize RNA Pol II core promoter elements.

Our results strongly suggest that the lack of DNA binding of a TBP-TAF_I48 dimer is sufficient to abolish transcriptional activation. However, TBP interacts directly with class II basal factors, TFIIA and TFIIB, and the recruitment of these factors by TBP or TFIID is required to assemble an active RNA Pol II initiation complex (*18*). Therefore, TAF_I48 may also disrupt such class-specific interactions. As a test of this idea, TAF_I48 or TBP-TAF_I48 complexes were tethered to an affinity resin by way of a FLAG-epitope tag on TAF_I48. Radiolabeled TFIIA or TFIIB was incubated with these complexes, and the proteins bound to the resin were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Fig. 4C). TFIIA does not interact directly with TAF_I48, but bound efficiently to the TBP-TAF_I48 resin. By contrast, TFIIB failed to bind either TAF_I48 or the TAF_I48-TBP complex. We also performed binding assays in which TBP-TFIIB complexes tethered to a resin were mixed with radiolabeled TAF_I48. Under these conditions, TAF_I48 did not bind efficiently to the TBP-TFIIB resin. In contrast, radiolabeled TFIIA bound efficiently to the TFIIB-TBP dimeric complex although it did not interact with TFIIB alone (Fig. 4C). Thus, the interaction of TAF_I48 with TBP precludes TFIIB from binding to TBP. It is possible that TAF_I48 and TFIIB interact with overlapping surfaces on TBP (*19*). Because TFIIA can bind the TBP-TAF_I48 complex, only a subset of interactions between TBP and class II factors appears to be affected.

Here we have studied both the promoter selectivity and UBF binding properties of TAFs associated with the RNA Pol I factor, SL1. We have shown that the presence of TAF_I48 in the SL1 complex is required to mediate transcriptional activation by UBF. Strictly speaking, TAF_I48 should be considered as a slightly different kind of coactivator because it is also required for transcription even in the absence of the activator UBF. For example, TAF_I48 may help position TAF_I110 within the SL1 complex in order to produce an active initiation complex. It is also likely that the TAF_I48 interaction with UBF will further stabilize the weak binding of TAF_I110 to elements of the promoter. Our experiments suggest that both TAF_I63 and TAF_I110 can make DNA contacts when in the SL1 complex. However, unlike a previous in vitro study, our in vivo studies failed to detect any direct DNA binding or contact with TAF_I48 (*20*).

Our results also indicate that a TBP-TAF_I48 complex is unable to bind to TATA boxes. The binding of TAF_I48 to TBP appears to be sufficient to block or conformationally alter the DNA binding surface of TBP in a manner that prevents recognition of the TATA-box element, consistent with the finding that SL1 fails to bind DNA containing TATA sequences (*12*, *21*). Therefore, different TBP-TAF complexes may mediate class-specific protein-protein interactions essential for the formation of active initiation complexes. For example, the inability of SL1 to transcribe RNA Pol II promoters may be due, in part, to the incompatibility between subunits of SL1 and interactions with other class-specific components of the transcription machinery (*22*, *23*). Thus, the promoter-selective and class-specific properties of different TBP-TAF complexes are reminiscent of bacterial σ-factors (*24*). Distinct TBP-TAF complexes can be viewed as multisubunit σ-factors that bind class-specific core promoter elements and interact with the transcriptional initiation complex to direct promoter-specific transcription. Future studies of TAFs, in both the SL1 and the TFIID complexes, should provide more detailed mechanisms responsible for promoter specificity and gene regulation.

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26. RNA Pol I was purified from HeLa nuclear extract with the following modifications (*9*). Briefly, after heparin agarose chromatography, RNA polymerase fractions (100 mM NaCl) were applied to a DEAE-CL6B ion-exchange column. RNA Pol I activity, eluted at 200 mM NaCl, was size fractionated on an S-300 gel filtration column before chromatography on a MonoS column. RNA Pol I activity elutes at 250 mM NaCl.
27. SL1 complexes were assembled as described with the following modifications (*11*). The triple SL1 complex lacking TAF_I48 was built on hemaglutinin (HA)–tagged TAF_I110 immobilized on protein A–Sepharose resin conjugated with anti-HA. After assembly, stable complexes were eluted with peptides (10 mg/ml) corresponding to the HA epitope. For footprint analysis, polyhistidine-tagged TAF_I63 (*11*) was renatured while still bound to Ni^{2+}-resin. The resin was then incubated with TBP (*11*). After the free TBP was washed away, the dimeric complexes were eluted with 500 mM imidazol {in TM buffer [25 mM tris(hydroxylmethyl)-aminomethane, pH 8; 12.5 mM $MgCl_2$, 100 mM NaCl, 10% (v/v) glycerol]}. After dialysis, the complexes were incubated with HA-110 immobilized on protein A–Sepharose cross-linked to anti-HA in the absence or presence of FLAG-tagged TAF_I48 [which was purified under native conditions from baculovirus-infected SF9 cell extracts and subsequently eluted with peptide (*10*)] to form a triple SL1 complex or complete rSL1 complex, respectively. The immobilized complexes were incubated for 1 hour at room temperature with the labeled promoter fragments that had been preincubated for 30 min with rUBF (*12*). After the unbound DNA was washed away, protein-DNA complexes were subjected directly to DNase I digestion (*15*, *25*). Reactions using endogenous, immunopurified holo SL1 from HeLa nuclear extract (*9*) were performed similarly.
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