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$TAF_{II}250$ Is a Bipartite Protein Kinase That Phosphorylates the Basal Transcription Factor RAP74

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Summary

Some TAF subunits of transcription factor TFIID play a pivotal role in transcriptional activation by mediating protein–protein interactions, whereas other TAFs direct promoter selectivity via protein–DNA recognition. Here, we report that purified recombinant $TAF_{II}250$ is a protein serine kinase that selectively phosphorylates RAP74 but not other basal transcription factors or common phosphoacceptor proteins. The phosphorylation of RAP74 also occurs in the context of the complete TFIID complex. Deletion analysis revealed that $TAF_{II}250$ contains two distinct kinase domains each capable of autophosphorylation. However, both the N- and C-terminal kinase domains of $TAF_{II}250$ are required for efficient transphosphorylation of RAP74 on serine residues. These findings suggest that the targeted phosphorylation of RAP74 by $TAF_{II}250$ may provide a mechanism for signaling between components within the initiation complex to regulate transcription.

Introduction

Production of mRNA by RNA polymerase II is directed by a multiprotein complex of some 50 polypeptides that must be assembled at promoter sequences of specific genes targeted for activation (for reviews, see Zawel and Reinberg, 1993; Tjian and Maniatis, 1994). In the past few years, many components of the transcription machinery have been purified and biochemically characterized. These include enhancer binding activators, subunits, and accessory cofactors of RNA polymerase II, as well as a slew of basal transcription factors (TFIIA, -B, -D, -E, -F, and -H) that are necessary to direct accurate and regulated initiation of transcription. A key component of the transcriptional initiation complex responsible for integrating and interpreting gene regulatory signals is the basal factor TFIID, which is composed of the TATA binding protein, TBP, and eight or more associated subunits called TAFs (Dynlacht et al., 1991; Tanese et al., 1991). Analysis of RNA polymerase II transcription in mutant cell lines bearing a temperature-sensitive TAF suggested that TAFs may function to direct activation of select genes in vivo (Sekiguchi et al., 1991; Ruppert et al., 1993; Wang and Tjian, 1994). Some TAFs are able to serve as coactivators that directly contact enhancer-bound activators to modulate gene-specific transcription (Goodrich et al., 1993; Hoey et al., 1993; Chen et al., 1994; Thut et al., 1995), whereas other TAFs in the complex are responsible for promoter recognition and DNA binding (Verrijzer et al., 1995). Thus, the TFIID complex may serve as a relay station sending messages between activators and the basal machinery.

Despite the rapid progress that has been made in identifying the essential components of the eukaryotic transcriptional apparatus, many questions regarding the mechanisms of regulation remain unknown. For example, although we have documented direct interactions between activators and TAFs as an important step during the process of transcriptional activation, how TAFs transduce signals to other basal factors and to RNA polymerase II in order to trigger the initiation of RNA synthesis remains unclear. In its simplest form, the binding of TAFs to select basal factors may be sufficient to recruit and stabilize the transcription machinery at the promoter. Indeed, $TAF_{II}40$ can bind to TFIIB (Goodrich et al., 1993) and $TAF_{II}250$ was recently shown to contact RAP74, the large subunit of TFIIF (Ruppert and Tjian, 1995). Because TFIIF is also responsible for binding directly to RNA polymerase II (Sopta et al., 1985; Burton et al., 1986, 1988), it seemed reasonable to postulate that one mode of transcription activation could occur via a cascade of protein–protein interactions starting with activators contacting TAFs and ending with TAFs recruiting TFIIB, TFIIF, and RNA polymerase II (Goodrich et al., 1993; Ruppert and Tjian, 1995).

However, it seemed unlikely that this passive model would account for all the different activation events that have thus far been identified. For example, not all activators act at the level of preinitiation complex formation. Some potent regulators of transcription are thought to act at later stages along the transcription cycle, such as promoter clearance (Narayan et al., 1994), reinitiation of stalled RNA polymerase complexes, and elongation (for review, see Lis and Wu, 1993). Also, only a subset of the TAFs have, thus far, been shown to serve as coactivators that directly contact activation domains. Thus, other subunits and subdomains of the TFIID complex may provide as yet unspecified functions important for transcription. In particular, it has become of interest to determine whether any of the TAFs are capable of catalyzing specific enzymatic reactions that might contribute to mechanisms of transcriptional activation. The notion that components of the transcriptional apparatus may carry out enzymatic activities is not entirely unfounded since it has been reported that various subunits of the basal factor TFIIH catalyze helicase and/or kinase activities important for promoter clearance (Lu et al., 1992; Feaver et al., 1993; Schaeffer et al., 1993; Serizawa et al., 1993; Goodrich and Tjian, 1994). In addition, some cofactors that are found associated with RNA polymerase II (i.e., SRBs) are also thought to be kinases (Liao et al., 1995). If indeed individual TAFs or the assembled TFIID complex were capable of intrinsic enzymatic function, such activities may play an important role in the

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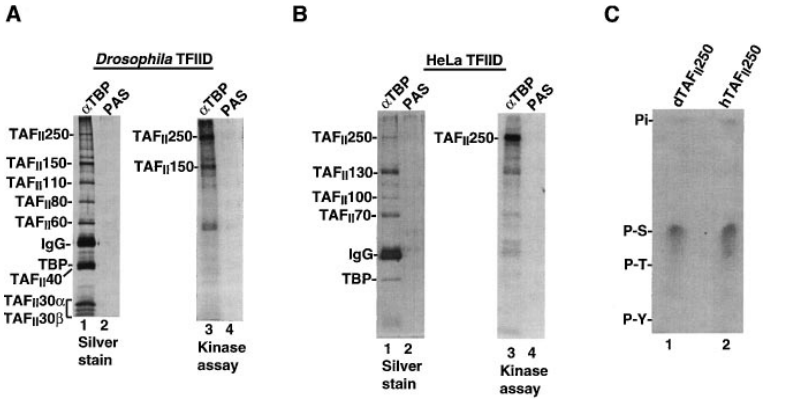

Figure 1. A Protein Kinase Is Associated with TFIID

(A) Drosophila TFIID was immunopurified by anti-TBP antibodies (coupled to protein sepharose beads) from nuclear extracts of 0–12 hours embryos and subjected to an in vitro kinase assay (see Experimental Procedures). As a control an identical process was performed with protein A sepharose beads (PAS) in the absence of the antibodies. The reactions were resolved on SDS–PAGE followed by silver staining (lanes 1 and 2) and autoradiography of the same gel (lanes 3 and 4).

(B) A similar in vitro kinase assay as in (A) was performed with HeLa TFIID that was affinity purified by human TBP antibodies from phosphocellulose (PC) 1 M NaCl fraction of HeLa nuclear extract.

(C) Phospho-amino acid analysis of phosphorylated Drosophila and human $TAF_{II}250$ ($dTAF_{II}250$ and $hTAF_{II}250$, respectively). The positions of standard phosphoamino acids (P-S= phosphoserine; P-T= phosphothreonine; P-Y= phosphotyrosine) and the free phosphate (Pi) are indicated.

mechanisms that direct communication between activators and the transcriptional apparatus, possibly through a cascade of phosphorylation events.

In this report, we have assayed affinity-purified endogenous TFIID for protein kinase activity. Purified recombinant TAF proteins were also tested for their ability to catalyze phosphotransferase activities. To discriminate between contaminating kinases associated with TFIID and intrinsic activities of TAFs, we have subjected candidate subunits to stringent purification procedures including high resolution SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by renaturation. A search for protein kinase substrates with specificity that might indicate a functionally relevant interaction was also undertaken. In addition, we have attempted to map the kinase domains of the TAF responsible for phosphorylation. Our results suggest that at least one TAF is able to catalyze covalent modification of basal transcription factors within the initiation complex and that this kinase activity may contribute to a novel mechanism for regulating transcription.

Results

A Protein Kinase Is Associated with TFIID

To test whether one or more subunits of TFIID might function as an enzyme capable of phospho-transferase activity, we subjected immunopurified TBP/TAF complexes isolated from Drosophila nuclear extracts to an in vitro kinase reaction followed by SDS–PAGE analysis. Of the nine subunits present in Drosophila TFIID (dTFIID), the two largest subunits, $TAF_{II}250$ and $TAF_{II}150$, were found to be efficiently phosphorylated (Figure 1A, lanes 1 and 3). No kinase activity was detected in control reactions carried out with mock immunoprecipitates isolated in the absence of TBP antibodies (Figure 1A, lanes 2 and 4). Kinase assays performed with immunopurified human TFIID (hTFIID) from HeLa cells also resulted in the phosphorylation of $TAF_{II}250$ (Figure 1B, lanes 1 and 3). A human homologue of Drosophila $TAF_{II}150$ has thus far not been identified in immunopurified TFIID, which may account for the lack of phosphorylated human $TAF_{II}150$ ($hTAF_{II}150$) in these reactions. These immunopurifications were carried out in the presence of ethidium bromide (400 μg/ml) to disrupt potential protein–DNA interactions. In addition, repeated washing of the immunopurified TFIID complex with high salt failed to remove the associated kinase(s) responsible for phosphorylating $TAF_{II}250$ (data not shown). Phosphoamino acid analysis of the Drosophila and human $TAF_{II}250$ subunits ($dTAF_{II}250$ and $hTAF_{II}250$, respectively) revealed that phosphorylation occurs predominantly at serine residues (Figure 1C). These experiments suggest that a serine protein kinase is either tightly associated with TFIID or one of its subunits is intrinsically a kinase.

$TAF_{II}250$ Is a Protein Kinase

The ability of highly purified TFIID to catalyze phosphorylation suggests that one or more of its integral subunits may be responsible for the observed kinase activity. To detect putative protein kinase activity catalyzed by a TAF subunit of TFIID, we performed a denaturation/renaturation autophosphorylation assay. This type of assay was adopted to discriminate between intrinsic TAF-mediated kinase activity versus contaminating kinases copurifying with TFIID. First, we immunopurified TFIID from a HeLa phosphocellulose fraction enriched for TFIID, and then the TAF/TBP subunits were subjected to SDS–PAGE followed by transfer to a nitrocellulose membrane. The immobilized proteins were subsequently denatured with guanidine-HCl and renatured in kinase buffer containing [γ-^{32}P]ATP to detect autophosphorylation of individual subunits. Under these stringent conditions, the largest subunit of the TFIID complex, $TAF_{II}250$, was efficiently autophosphorylated (Figure 2A, lane 1). The identity of $TAF_{II}250$ was confirmed by Western blot analysis using monoclonal $TAF_{II}250$ antibody 6B3 (Figure 2A, lane 2). In addition to $TAF_{II}250$, two other polypeptides (approximate molecular masses, 68 and 40 kDa) were also found to be phosphorylated in this assay. However, neither of these two polypeptides corresponded in size to any of the known TAFs. It is possible that they are proteolytic breakdown products of $TAF_{II}250$ that retained kinase activity (see

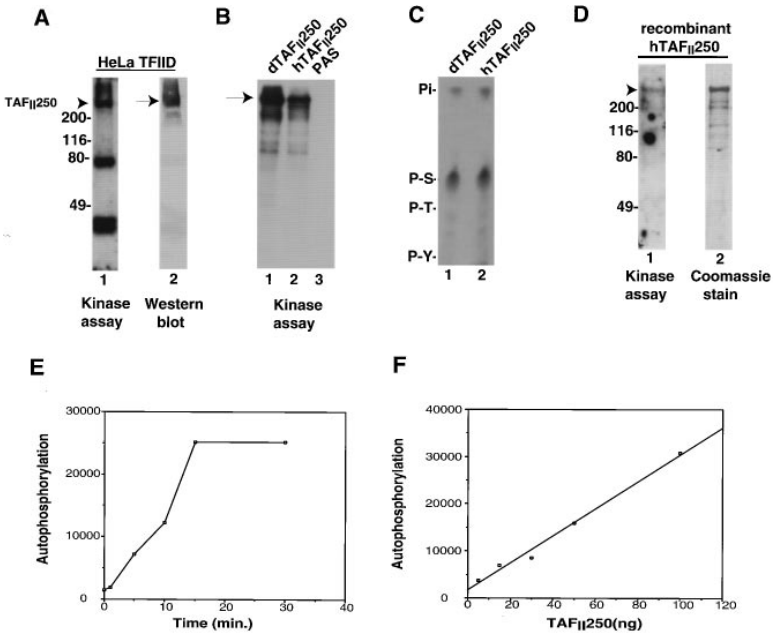

Figure 2. $TAF_{II}250$ Catalyzes an Intrinsic Kinase Activity

(A) TFIID complex was immunopurified from HeLa PC 1.0 M NaCl fraction by anti-TBP antibodies, resolved on SDS–PAGE and transferred onto nitrocellulose membrane. The immobilized TBP and TAF polypeptides were renatured in kinase buffer followed by a kinase reaction in the presence of [γ-^{32}P]ATP (kinase assay, lane 1). The same membrane was also subjected to Western blot analysis with anti-$TAF_{II}250$ monoclonal antibody 6B3 (lane 2). The position of $hTAF_{II}250$ is indicated by the arrowheads, and the position of molecular weight markers is indicated on the left and given as kDa throughout the paper.

(B) Drosophila $TAF_{II}250$ ($dTAF_{II}250$, lane 1) and human $TAF_{II}250$ ($hTAF_{II}250$, lane 2) were expressed as HA-tagged proteins in the baculovirus expression system and purified by anti-HA antibodies immobilized on protein A sepharose beads. The purified proteins were used for an in vitro kinase assay followed by SDS–PAGE and autoradiography. As a control, an Sf9 extract containing $hTAF_{II}250$ was processed in the absence of antibody (PAS, lane 3).

(C) Phosphoamino-acid analysis of the autophosphorylated recombinant $dTAF_{II}250$ (lane 1) and $hTAF_{II}250$ (lane 2) proteins.

(D) Anti-HA immunopurified human $TAF_{II}250$ was resolved from contaminating kinases by SDS–PAGE, transferred onto nitrocellulose membrane, renatured, and subjected to the autophosphorylation reaction (lane 1). The Purified $TAF_{II}250$ protein used for this experiment is shown after Coomassie blue staining (lane 2). The arrow points towards full-length $hTAF_{II}250$.

(E) In vitro kinase reactions were performed for the indicated time points at 30°C using 50 ng of immunopurified recombinant $hTAF_{II}250$. The reactions were terminated by the addition of protein sample buffer followed by SDS–PAGE. The autophosphorylation was quantitated by phosphoimager, and the activity is represented by phosphoimager units.

(F) Increasing amounts of immunopurified $hTAF_{II}250$ were used in an in vitro autophosphorylation assay for 10 min at 30°C, and the autophosphorylated $hTAF_{II}250$ bands were quantitated by phosphoimager.

below) but failed to be recognized by the $TAF_{II}250$ monoclonal antibody. Alternatively, there might be additional protein kinases associated with TFIID that are present in substochiometric amounts in the complex and are, therefore, not easily detected by conventional protein staining procedures. To confirm that the incorporation of label is due to covalent modification and not merely a result of binding labeled nucleotide, the phosphorylated $TAF_{II}250$ immobilized on filters was subjected to phosphoamino acid analysis and radiolabeled phosphoserine was detected (data not shown).

To further substantiate that $TAF_{II}250$ has an intrinsic kinase activity, epitope-tagged recombinant full-length Drosophila and human proteins were expressed in Sf9 cells, purified to homogeneity by affinity chromatography, and subjected to the in vitro kinase assay (Figure 2B). Both purified recombinant d- and $hTAF_{II}250$ became efficiently autophosphorylated on serine residues in vitro (Figure 2C and data not shown). The intrinsic autophosphorylation activity of purified recombinant $hTAF_{II}250$ was further confirmed by a kinase assay performed after SDS–PAGE separation and transfer to nitrocellulose (Figure 2D). We also confirmed that, under these denaturation/renaturation assay conditions, no labeled $TAF_{II}250$ can be observed if [α-^{32}P]ATP is used in place of [γ-^{32}P]ATP (data not shown). These findings taken together strongly support the notion that at least one subunit of TFIID, $TAF_{II}250$, is a protein serine kinase capable of autophosphorylation.

To characterize the autophosphorylation activity of $TAF_{II}250$, time course and dose–response curves were performed (Figures 2E and 2F). These studies show that the linear range of the autophosphorylation activity is from 1 to 15 min at 30°C and that this activity is dose dependent in a linear manner when the reaction is incubated for 10 min. We also found that ATP, dATP, and GTP, but not CTP and UTP, can be utilized by $TAF_{II}250$ (data not shown).

RAP74 Is Specifically Phosphorylated by $TAF_{II}250$ In Vitro

The intrinsic kinase activity of $TAF_{II}250$ raised the possibility that this subunit of TFIID might also be capable of phosphorylating other protein substrates. Within the TBP/TAF complex, we have found that the other TAF subunits and TBP are not phosphorylated by $TAF_{II}250$ (see Figure 1), with the possible exception of $TAF_{II}150$. However, when we directly tested the ability of $TAF_{II}250$ to phosphorylate purified $dTAF_{II}150$, we failed to detect any phosphotransferase activity, although autophosphorylation of $TAF_{II}250$ was observed (Table 1). It is possible, therefore, that the phosphorylation of $TAF_{II}150$ requires an intact TFIID complex or, alternatively, is catalyzed by some contaminating and unrelated kinase.

Next, we tested the potential transphosphorylation activity of $TAF_{II}250$ by using a variety of standard serine/threonine substrates such as histone H1, casein, myelin basic protein, and the RNA polymerase II C-terminal domain (CTD) under standard kinase reaction conditions. Surprisingly, none of these substrates was phosphorylated by $TAF_{II}250$ (Table 1). To examine the ability of $TAF_{II}250$ to transphosphorylate potentially relevant

Table 1. Substrate Selectivity of $TAF_{II}250$ Kinase

Substrate	TAF_{II} 250 Kinase Activity
Histone H1	−
Casein	−
Myelin basic protein	−
RNA pol II CTD tail	−
$dTAF_{II}$ 150	−
TBP	−
dTFIIA, large subunit	+
dTFIIA, small subunit	−
TFIIB	−
TFIIE, p56 subunit	+
TFIIE, p34 subunit	−
TFIIF, RAP74 subunit	+++++
TFIIF, RAP30 subunit	−

substrates, we tested various other components of the RNA polymerase II transcriptional initiation complex. In vitro kinase reactions were carried out with recombinant basal transcription factors: TBP, TFIIA (large and small subunits), TFIIB, TFIIE (p56 and p34), and TFIIF (RAP74 and RAP30). As shown in Table 1 and Figure 3A, only the large subunit of TFIIF (RAP74) was efficiently phosphorylated by $TAF_{II}250$. We also detected some labeling of the large subunits of TFIIA and TFIIE, although the efficiency was 10 times less than phosphorylation of RAP74. The phosphorylation of RAP74 by $TAF_{II}250$ does not require the presence of RAP30 nor is it impeded by the presence of this small subunit of TFIIF (data not shown).

As an additional precaution against the remote possibility that a kinase fortuitously associated with affinity-purified recombinant $hTAF_{II}250$ from Sf9 cells is responsible for phosphorylation of RAP74, we have carried out a modified version of the denaturation/renaturation assay adapted for transphosphorylation. First, we subjected affinity-purified recombinant $hTAF_{II}250$ to SDS–PAGE separation followed by transfer to nitrocellulose. After denaturation of the immobilized $hTAF_{II}250$ with guanidine-HCl and renaturation, the region of the membrane containing the $hTAF_{II}250$ protein was excised and incubated with recombinant RAP74 purified from E. coli. Under these stringent assay conditions, we observed significant phosphorylation of RAP74 by autoradiography after SDS–PAGE (Figure 3C). A control reaction in which purified RAP74 was incubated with a slice of control membrane showed no transfer of label to the protein substrate. Phosphoamino acid analysis of RAP74 indicates that phosphorylation occurs predominantly on serine residues (Figure 3B). These experiments suggest that $TAF_{II}250$ is a serine kinase with a high degree of substrate specificity for RAP74.

Interestingly, RAP74 was recently shown to interact directly with $hTAF_{II}250$ (Ruppert and Tjian, 1995), suggesting that the phosphorylation of RAP74 might be of functional importance. To provide supporting evidence that RAP74 may be a relevant substrate for phosphorylation by $TAF_{II}250$, we have also tested the ability of native TFIID to phosphorylate RAP74. In the TFIID complex, $TAF_{II}250$ contacts TBP as well as many other TAFs. We therefore wanted to see whether $TAF_{II}250$ is accessible in the context of the TFIID complex to serve as a kinase

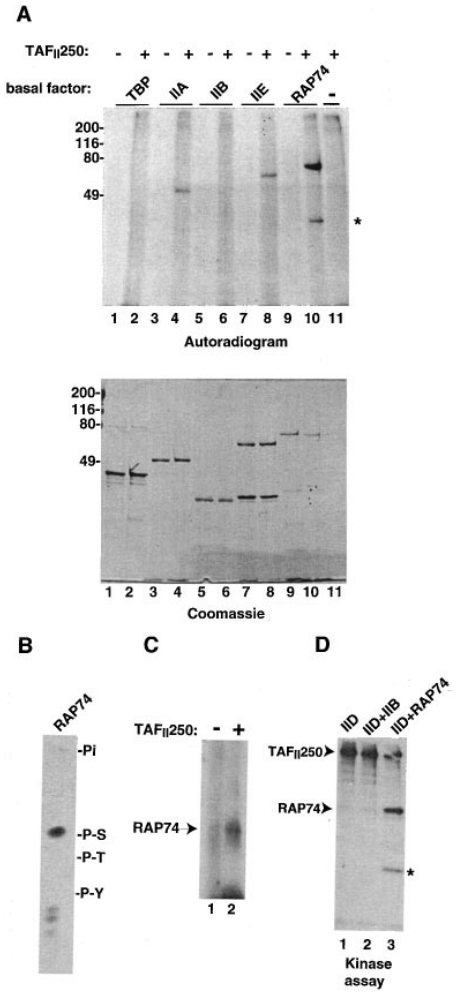

Figure 3. $TAF_{II}250$ Selectively Phosphorylates RAP74

(A) In vitro phosphorylation of basal transcription factors purified from E. coli extracts by immunopurified $hTAF_{II}250$ (lanes 2, 4, 6, 8, 10, and 11). As a control, reactions were performed in the absence of $hTAF_{II}250$ (lanes 1, 3, 5, 7, and 9). The proteins were resolved on SDS–PAGE followed by Coomassie blue staining (lower panel) and autoradiography for 5 min (upper panel). Lanes 1 and 2, TBP; lanes 3 and 4, Drosophila TFIIA, large and small subunits; lanes 5 and 6, TFIIB; lanes 7 and 8, TFIIE, p56 and p34; lanes 9 and 10, RAP74. The asterisk indicates a copurified RAP74 breakdown product. The amount of $hTAF_{II}250$ used in the experiment was too low (5 ng) to be detected by autophosphorylation and Coomassie blue staining.

(B) Phosphoamino acid analysis of RAP74 phosphorylated by $hTAF_{II}250$.

(C) Phosphorylation of RAP74 by gel purified $TAF_{II}250$. Immunopurified $hTAF_{II}250$ was resolved by SDS–PAGE and immobilized on nitrocellulose membrane. After staining with ponceau S, the $hTAF_{II}250$ band was excised, renatured, and subjected to a kinase reaction in the presence of purified RAP74 (lane 2). As a control, RAP74 was subjected to a kinase reaction in the absence of $hTAF_{II}250$ (lane 1). The reactions were stopped by the addition of protein sample buffer followed by SDS–PAGE and autoradiography.

(D) Phosphorylation of RAP74 by TFIID complex. TFIID complex was immunopurified from PC 1.0 M NaCl fraction followed by an in vitro kinase reaction in the absence (lane 1) or presence of purified recombinant TFIIB (lane 2) and RAP74 (lane 3). The positions of phosphorylated $TAF_{II}250$ and RAP74 are shown. A phosphorylated RAP74 breakdown product is indicated by the asterisk.

able to phosphorylate RAP74. For this purpose, we have immunopurified hTFIID and subjected the TBP/TAF complex to in vitro kinase reactions in the presence of recombinant RAP74 or TFIIB. As seen in Figure 3D, RAP74 is efficiently phosphorylated by the TFIID complex, whereas no phosphorylation of TFIIB was observed, suggesting that the specific covalent modification of RAP74 by $TAF_{II}250$ can occur in the context of the TFIID complex and may play a role in the regulation of transcription.

A Kinase Domain Resides within the N-Terminal Portion of $TAF_{II}250$

A comparison of amino acid sequences from many different eukaryotic protein kinases identified a series of conserved domains common to most kinases (Hanks et al., 1988). An initial computer-assisted search for protein kinase consensus sequences failed to reveal any obvious homologies between TAFs and kinases. However, the observed kinase activity of $TAF_{II}250$ prompted us to reexamine the amino acid sequence of $TAF_{II}250$ for potential sequence similarity to eukaryotic protein kinases. A more detailed visual comparison of $TAF_{II}250$ to kinase domains revealed several patches of amino acid sequences with significant but weak homology to the protein kinase family (Figure 4A). The best match to multiple kinase homologies was found within the N-terminal region of both d- and $hTAF_{II}250$. This finding raised the possibility that a kinase domain may be located within the N-terminal domain of $TAF_{II}250$. To test this possibility, two N-terminal fragments corresponding to the first 1054 and 643 amino acids of $hTAF_{II}250$ were expressed in Sf9 cells, immunopurified, and subjected to in vitro kinase reactions. As shown in Figure 4B (lanes 2 and 3), these truncated proteins retained the ability to catalyze autophosphorylation. To confirm that the kinase activity associated with the N-terminal regions of $hTAF_{II}250$ is intrinsic to this domain, the denaturation/renaturation assay was performed with these two truncated proteins. Significant kinase activity was recovered even under these stringent autophosphorylation conditions (Figure 4B, lanes 4 and 5), suggesting that a kinase domain is indeed located within the N-terminal region of $TAF_{II}250$. Phosphoamino acid analysis of the phosphorylated N643 protein confirms that serine residues are the predominant phosphate acceptors (data not shown). To further map the N-terminal kinase domain (NTK) of $hTAF_{II}250$, additional deletion mutants were expressed as glutathione S-transferase (GST) fusion proteins in Sf9 cells (Figures 4C and 4E). The ability of these deletion mutants to phosphorylate the RAP74 substrate was analyzed under standard kinase reaction conditions. These experiments map a minimal kinase domain of $TAF_{II}250$ capable of efficient autophosphorylation and transphosphorylation to amino acid residues 1–434 (Figures 4C and 4E). It also appears that the region between residues 1–161 carries sequences important for the transphosphorylation of RAP74, as deletions in this domain abrogated RAP74 phosphorylation (Figure 4C, compare lanes 3 and 4) but did not effect autophosphorylation.

We also expressed this NTK of $TAF_{II}250$ in E. coli as a GST fusion protein. The levels of expression of this protein were very low in bacterial cells. Nevertheless, the purified protein from E. coli was capable of autophosphorylation and transphosphorylation on serine residues of RAP74 (Figure 4D and data not shown). However, its specific activity was significantly lower than $TAF_{II}250$ purified from eukaryotic cells, raising the interesting possibility that the kinase activity of $TAF_{II}250$ might itself be regulated by some form of posttranslational modification.

A Second Distinct Kinase Domain Is Located at the C-Terminal Region of $TAF_{II}250$

Because $TAF_{II}250$ is a very large protein with a potential for encoding many different functional domains, other regions of this protein were also tested for kinase activity. We began by constructing a deletion mutant of $hTAF_{II}250$ lacking the NTK (ΔN554). This truncated product of 1399 amino acids was expressed in Sf9 cells, immunopurified, and assayed for kinase activity. To our surprise, this truncated protein was significantly active for autophosphorylation (Figure 5A). Kinase activity was also found to be catalyzed by a similar C-terminal domain of $dTAF_{II}250$ (ΔN660; Figure 5A). To further characterize the kinase activity associated with the C-terminal domain of $hTAF_{II}250$, several additional deletion mutants were expressed as GST fusion proteins in Sf9 cells. After purification, these mutant proteins were subjected to the denaturation/renaturation assay (Figure 5B). This analysis revealed that the observed autophosphorylation is intrinsic to amino acid sequences located within the C-terminal 468 amino acid residues of $hTAF_{II}250$ (CTK). Interestingly, the amino acid sequence of this putative CTK shares no apparent homology to other known protein kinases including the NTK of $TAF_{II}250$, suggesting that it might represent a novel class of protein kinase domain.

Efficient Phosphorylation of RAP74 by $TAF_{II}250$ Requires Both Kinase Domains of $TAF_{II}250$

To determine which of the kinase domains of $TAF_{II}250$ is responsible for the transphosphorylation of RAP74, the ability of each domain to phosphorylate this heterologous substrate was determined independently. First, we tested the NTK for its ability to phosphorylate RAP74. For these studies, we used a series of C-terminal truncations that progressively remove larger portions of the protein. All of these truncated NTK proteins were significantly impaired in their ability to phosphorylate RAP74 when compared with the full-length $TAF_{II}250$ (Figures 6A and 6B, lanes 2 and 4). Interestingly, the absence of RAP74 interaction domain has no significant effect on the residual RAP74 phosphorylation by the N-domain. These results suggest that, although the NTK can autophosphorylate efficiently, it is compromised in its ability to transphosphorylate RAP74.

Next, we used a number of deletion mutants lacking the NTK but containing the CTK. One of these mutant proteins (ΔN700) includes the RAP74 binding domain, whereas two other mutants we tested (ΔN1162 and ΔN1206) lack part of RAP74 binding domain of $TAF_{II}250$

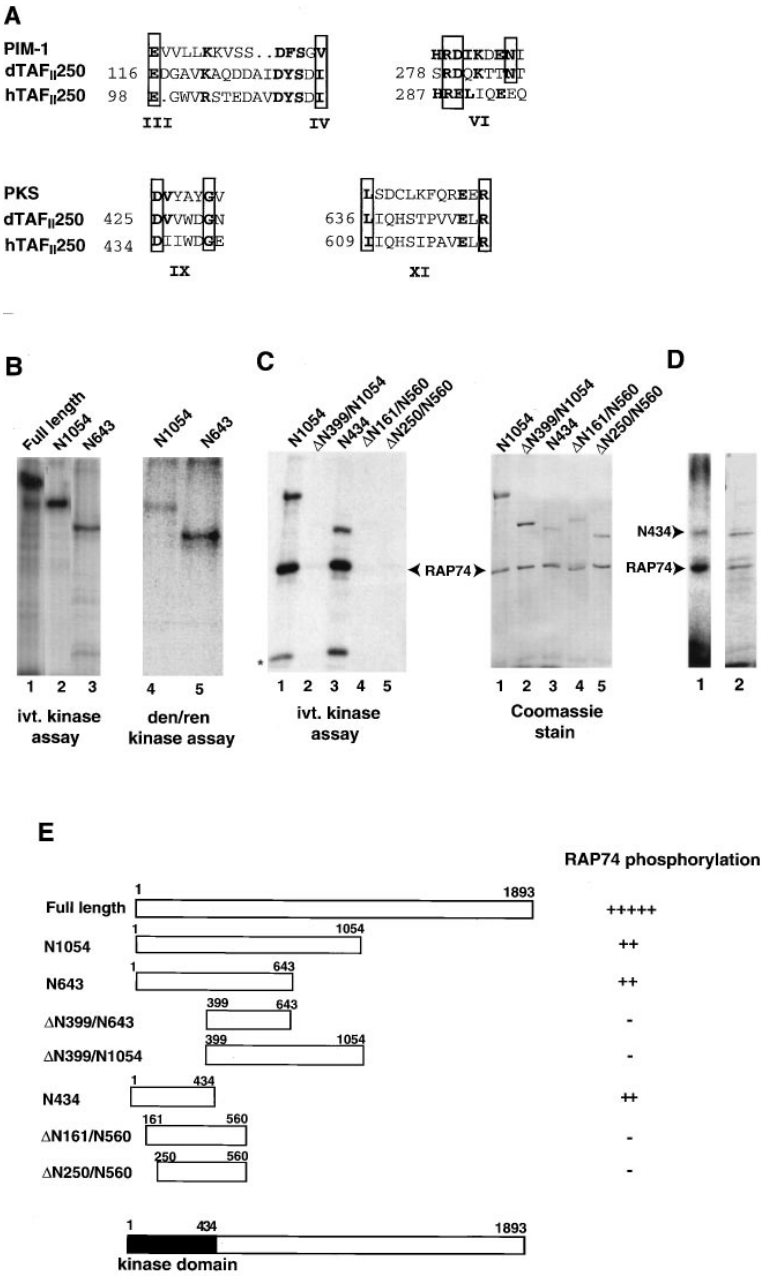

Figure 4. A Catalytic Kinase Domain Resides at the N-terminus of TAF$_{II}$250

(A) Sequence homology of the amino-terminus of Drosophila and human TAF$_{II}$250 to protein kinases. The best homologous regions include kinase subdomains III, IV, and VI of PIM-1 protein kinase and regions IX and XI of PKS (Hanks et al., 1988). The conserved amino acids are boxed and additional homologies are in bold.

(B) A kinase activity is associated with the hTAF$_{II}$250 N-terminus. HA-epitope tagged wild type and C-terminal deletion mutants of hTAF$_{II}$250, N1054, and N643 were expressed in Sf9 cells, purified by anti-HA antibodies and subjected to an in vitro kinase assay followed by SDS–PAGE and autoradiography (lanes 1–3). Purified N1054 and N643 proteins were subjected to the denaturation/renaturation autophosphorylation assay (lanes 4 and 5).

(C) Mapping of the N-terminal kinase domain (NTK). N- and C-terminal truncations of the N-terminal half of hTAF$_{II}$250 (1–1054) were constructed and expressed in Sf9 cells as GST fusion proteins. The proteins were affinity purified by binding to GST beads and analyzed by Coomassie staining (right panel) and for RAP74 phosphorylation by in vitro kinase assay (ivt. kinase assay, left panel). The position of RAP74 is shown by arrowheads.

(D) The 1–434 NTK was expressed in E.coli as a GST fusion protein, purified by GST beads (Coomassie stain, lane 2), and tested in an in vitro kinase assay in the presence of RAP74 (lane 1). The positions of N434 and RAP74 are shown. (E) Schematic representation and summary of the kinase activities of full-length and various portions of hTAF$_{II}$250 N-terminus. The activity of wild-type and mutant proteins is shown by their ability to phosphorylate RAP74. The position of the truncated proteins is with respect to the full-length protein of 1893 amino acid residues (Ruppert et al., 1993) and is indicated by open bars. The minimal NTK is indicated by the solid portion in the full-length protein shown at the bottom.

(Ruppert and Tjian, 1995). Phosphorylation of RAP74 by ΔN1206 or ΔN1162 was virtually abolished (Figure 6, lanes 6 and 7). By contrast, ΔN700, which retains the RAP74 binding domain of TAF$_{II}$250, was able to phosphorylate RAP74, albeit rather weakly when compared with the full-length TAF$_{II}$250 protein (Figure 6, lane 5). These results suggest that efficient phosphorylation of RAP74 by TAF$_{II}$250 requires both the NTK and CTK of TAF$_{II}$250. Moreover, these findings suggest that the residual transphosphorylation activity of the CTK is dependent on the RAP74 binding domain, consistent with the notion that binding of the substrate protein by TAF$_{II}$250 may contribute to the specificity of phosphorylation.

Discussion

The finding that TAF subunits of the basal factor TFIID are required to mediate transcriptional activation prompted us to initiate a systematic biochemical characterization of these transcription factors. Our previous studies established that some TAFs contain coactivator domains that bind directly to activators (Chen et al., 1994; Sauer et al., 1995; Thut et al., 1995), while other subunits of TFIID are able to recognize and interact with specific DNA sequences in core promoters (Verrijzer et al., 1994, 1995). In addition, several TAFs have been shown to contact other components of the transcriptional machinery (Goodrich et al., 1993; Ruppert and

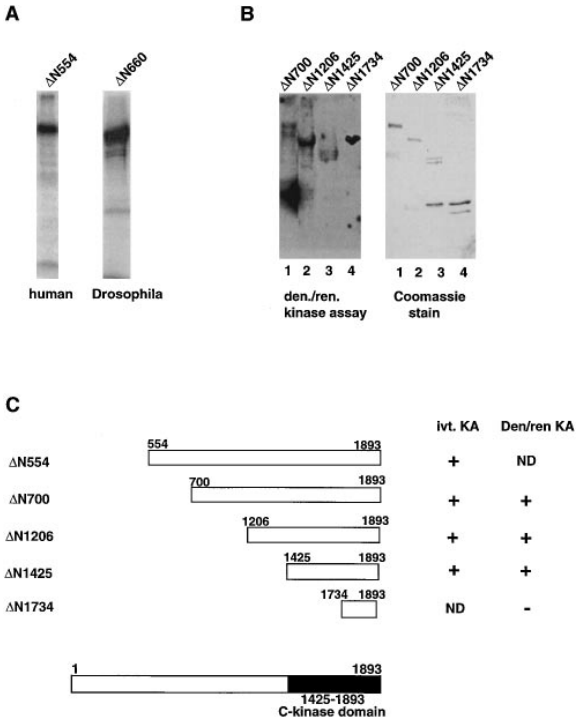

Figure 5. C-Terminal Kinase Domain

(A) A C-terminal portion of human (amino acid residues 554–1893) and Drosophila (amino acid residues 660–2068) $TAF_{II}250$ lacking the NTK were expressed in Sf9 cells, purified by anti-HA antibody (human) or anti-$dTAF_{II}250$ antibody (Drosophila) and subjected to the standard in vitro autophosphorylation assay.

(B) Mapping of the C-terminal kinase domain (CTK). N-terminal deletion mutant proteins of $hTAF_{II}250$ were expressed in Sf9 cells as GST fusion proteins, purified, and subjected to the denaturation/renaturation kinase assay (left panel). The protein preparations used for these experiment are shown in the right panel.

(C) Schematic representation and kinase activity of $hTAF_{II}250$ N-terminal truncations. The position of the truncated proteins is with respect to the full-length protein of 1893 amino acid residues (Ruppert et al., 1993) and is indicated by open bars. The relative position of the minimal CTK (indicated by the solid portion) in the full-length protein is shown at the bottom. ND indicates that kinase activity was not determined.

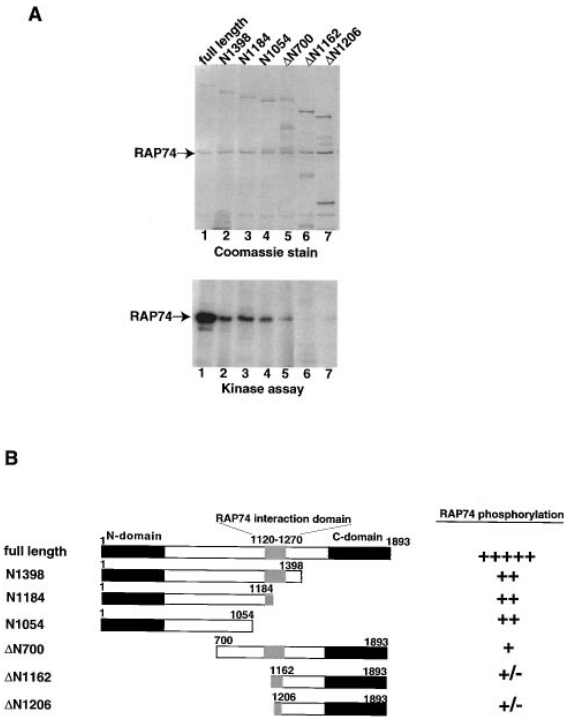

Figure 6. Phosphorylation of RAP74 by N- and C-Terminal Kinase Domains

(A) In vitro phosphorylation of RAP74 by various NTKs and CTKs of $TAF_{II}250$ that either contain (ΔN700, N1398) or lack part of the RAP74 interaction domain (ΔN1162, ΔN1206, and N1184) or are completely missing the RAP74 interaction domain (N1054) (Ruppert and Tjian, 1995). The autoradiogram of RAP74 phosphorylation is shown in the lower panel and the proteins used for the reactions are shown in the Coomassie blue stained SDS–PAGE in the upper panel.

(B) A schematic representation and summary of the transphosphorylation activity of wild-type and mutant $hTAF_{II}250$ proteins. RAP74 interaction domain and the NTK and CTK of $hTAF_{II}250$ are indicated.

Tjian, 1995). However, few TAFs have been characterized in detail, and most probably contain multiple functional domains with potentially novel activities that remain to be discovered. In this report, we have focused on the largest of the TFIID subunits, $TAF_{II}250$, in an effort to better understand its relationship to the other components of the transcriptional apparatus.

$TAF_{II}250$ is thought to serve as the core subunit of TFIID, providing specific interfaces for interaction with a variety of other TAFs as well as TBP. However, this core subunit is not likely to provide merely an inert scaffold for the assembly of TFIID. Instead, several lines of evidence suggest that $TAF_{II}250$ may carry out a variety of important functions typically attributed to TAFs. First, temperature-sensitive mutations of $TAF_{II}250$ are defective for transcriptional activation of certain genes in vivo and in vitro (Sekiguchi et al., 1991; Wang and Tjian, 1994), suggesting that it may serve as a coactivator for a subset of activators. Second, $TAF_{II}250$ has been found to cooperate with $TAF_{II}150$ in recognizing and binding specific promoter sequences, including initiator elements near the start site of transcription (Verrijzer et al., 1995; Hansen and Tjian, 1995). Most recently, a domain of $TAF_{II}250$ (amino acid residues 1120–1270) was shown to bind selectively to the large subunit, RAP74, of the basal factor TFIIF (Ruppert and Tjian, 1995). Now, we report that $TAF_{II}250$ is able to catalyze the transfer of phosphate from ATP to serine residues of RAP74. The protein kinase activity of $TAF_{II}250$ appears to be highly substrate specific, as it is unable to phosphorylate a variety of common phosphoacceptor proteins. Moreover, none of the other eight subunits of TFIID that are in contact with $TAF_{II}250$ appear to be targeted for phosphorylation by $TAF_{II}250$. Interestingly, in addition to RAP74, $TAF_{II}250$ is able to weakly phosphorylate the large subunits of TFIIA and TFIIE but not other components of the basal machinery. These findings are consistent with the notion that the phosphorylation of RAP74, and possibly TFIIA and TFIIE, by $TAF_{II}250$ might represent highly specific and potentially important steps during transcriptional regulation. It is, of course, possible

and perhaps even likely that there are other, as yet undetected, physiologically relevant substrates for the $TAF_{II}250$ kinase.

Although there is no direct evidence to indicate the functional role of RAP74 phosphorylation by $TAF_{II}250$ in transcription, our analysis of $TAF_{II}250$ deletion mutants, taken together with an independent study of $TAF_{II}250$ deletion mutants performed by Noguchi et al. (1994), supports a role for $TAF_{II}250$ kinase domains in vivo. Deletion of a region spanning part of the NTK (amino acid residues 166–352), in the context of mutants lacking the CTK, resulted in truncated proteins incapable of rescuing the temperature-sensitive phenotype of ts13 cells (Noguchi et al., 1994). Interestingly, a mutant lacking the CTK retained some ability to rescue ts13 cells, raising the possibility that this domain might be responsible for a regulatory function. These results, taken together, are consistent with the hypothesis that the kinase activity of $TAF_{II}250$ provides some important function during the process of transcriptional regulation in vivo.

It is not difficult to imagine that the selective covalent modification of one essential factor (i.e., RAP74) by another ($TAF_{II}250$) within the initiation complex could provide an efficient and specific mechanism for signaling between transcription factors during the process of activation. For example, it has been postulated that the phosphorylation of the RNA polymerase II CTD by TFIIH and/or kinases associated with RNA polymerase II (for review, see Conaway and Conaway, 1993) could serve as a trigger to induce the transcription complex to proceed from an initiation stage into the elongation phase. Phosphorylation of the CTD does not, however, appear to be a requirement for transcription elongation since RNA polymerase mutants lacking a CTD are active for transcription, at least in systems reconstituted in vitro with purified factors (Zehring and Greenleaf, 1990; Li and Kornberg, 1994). Likewise, it is conceivable that the phosphorylation of RAP74, which is an essential basal factor tightly associated with RNA polymerase II, could also help the transition from initiation to elongation. It has also been reported that TFIIH kinase can phosphorylate some of the basal factors (Ohkuma and Roeder, 1994); therefore, it may be that a cascade of phosphorylation events involving multiple kinases associated with the transcription complex (i.e., TFIIH/CAK, DNA PK, Cdk/cyclin, etc.) including $TAF_{II}250$ serves as a signal transduction pathway for communication between enhancer complexes, the TFIID complex, and the RNA polymerase II complex. However, it seems unlikely that phosphorylation of RAP74 will be a prerequisite to transcription elongation since transcription reactions carried out with TBP alone in the absence of $TAF_{II}250$ are competent for elongation. Thus, like the phosphorylation of CTD by TFIIH, the phosphorylation of RAP74 by $TAF_{II}250$ may be modulatory rather than essential for basal transcription.

An intriguing alternative possibility is that the phosphorylation of RAP74 by $TAF_{II}250$ may be a specialized event during the transcriptional activation process that is only required by a subset of promoters. For example, a number of genes are regulated at postinitiation events such as promoter clearance, antipausing, and elongation. In some cases, transcription complexes that have initiated but become stalled or paused a short distance downstream from the start site must be "induced" to continue and enter the elongation phase. Perhaps, a specialized class of activators that have evolved to direct transcriptional regulation at this stage of the reaction will require some specific signaling mechanism. One way to relay such an activation signal between activators bound to enhancers and stalled RNA polymerase/RAP74 complexes would be through phosphorylation of components within the transcribing complex. It is also intriguing that temperature-sensitive mutants of $TAF_{II}250$ have been isolated that block cell cycle progression ($G_1 \rightarrow S$) at the nonpermissive temperature (Sekiguchi et al., 1988, 1991). Although we have previously interpreted this finding to suggest that $TAF_{II}250$ functions as a coactivator required to mediate activation of genes important for cell cycle progression, it is conceivable that the kinase activity of $TAF_{II}250$ may play a more direct role in coupling transcription regulation to cell cycle control. It will, therefore, be of interest in the future to determine whether $TAF_{II}250$ kinase activity is indeed required to regulate the transcription of select genes and whether it has any part in more direct mechanisms that modulate cell cycle.

A preliminary deletion mapping of $TAF_{II}250$ functional domains unexpectedly revealed a novel bipartite kinase structure. A kinase domain of ~400 amino acid residues located at the N-terminus of $TAF_{II}250$ appears to be necessary to catalyze both autophosphorylation and transphosphorylation of RAP74. This NTK encompasses a region that displays a modest sequence similarity to consensus kinase domains. However, at present, we have not mapped the putative ATP binding or catalytic sites of $TAF_{II}250$ to ascertain the functional importance of this homology. We also identified an additional extended region of ~400 amino acids toward the C-terminus of $TAF_{II}250$ that is capable of autophosphorylation but shares no apparent homology to other kinases. However, efficient transphosphorylation of RAP74 requires the combined action of the NTK and CTK. It also appears that the RAP74 binding region of $TAF_{II}250$ may contribute to the efficiency and presumably the specificity of RAP74 phosphorylation. These properties of the $TAF_{II}250$ kinases are reminiscent of the RSK family of bipartite kinases (Bjørbæk et al., 1995), although the substrate specificity of $TAF_{II}250$ appears to be substantially more selective. It will be interesting to determine whether $TAF_{II}250$ kinase activity is in any way functionally linked to the signal transduction pathways of the RSK kinases.

A comparison of $TAF_{II}250$ amino acid sequences from Drosophila, human, and hamster revealed substantial structural similarities within the putative kinase domains and RAP74 binding regions. However, unlike $TAF_{II}250$ from these metazoan organisms, the yeast homologue that is 140 kDa (Reese et al., 1994; Poon et al., 1995) lacks both putative kinase domains as well as the RAP74 binding region. These findings lend further support to the idea that the role of $TAF_{II}250$ kinase is not likely to be at the level of basal transcription but instead may provide a mechanism dedicated for regulating specialized transcription units that may not be found in yeast.

It will be interesting to determine the structure and functional relationship between the two putative kinase domains of $TAF_{II}250$ and how they work coordinately to target phosphorylation of RAP74. Our studies also imply that there may be multiple mechanisms directing the regulation of transcription and that specific enzymatic activities associated with the transcriptional machinery may play a critical role in the signaling pathways that are required to integrate and interpret the myriad regulatory events necessary to control transcription in eukaryotes.

Experimental Procedures

Construction of Plasmids

The baculovirus transfer vectors encoding $hTAF_{II}250$ and truncated versions thereof—pbHAX-$hTAF_{II}250$, pbGST-$hTAF_{II}250\Delta$N700, ΔN1162, ΔN1206, ΔN1425, and ΔN1734—and the baculovirus transfer vector encoding the C-terminal part of $dTAF_{II}250$ (C180, renamed ΔN660) were described earlier (Ruppert et al., 1993; Weinzierl et al., 1993; Ruppert and Tjian, 1995).

C-Terminal Truncations of $hTAF_{II}250$ in Baculovirus Transfer Vectors

Baculovirus transfer vectors containing the hemagglutinine (HA)-tag, pbHAX-$hTAF_{II}250$N1054, N643, N554, and N434, were constructed by subcloning the NdeI/BamHI fragments derived from pETHAX-$hTAF_{II}250$N1054, N643, N554, and N434 (Ruppert and Tjian, 1995) into pVL1392HAX (Ruppert et al., 1993). Baculovirus transfer vectors containing the GST-tag, pbGST-$hTAF_{II}250$N1398, N1184, and N1054, were constructed by transferring the appropriate fragments from pETHAX-$hTAF_{II}250$N1398, N1184, and N1054 (Ruppert and Tjian, 1995) into pVL1392GST (Ruppert and Tjian, 1995).

N-Terminal/C-Terminal Truncations of $hTAF_{II}250$ in Baculovirus Transfer Vectors

Subcloning of the XbaI (cDNA position 1213)/ScaI (cDNA position 1943) and XbaI/HincII (cDNA position 3174) cDNA fragments into pVL1392GST (Ruppert and Tjian, 1995) yielded the constructs pbGST-$hTAF_{II}250\Delta$N399/N643 and pbGST-$hTAF_{II}250\Delta$N399/N1054. pbGST-$hTAF_{II}250\Delta$N161/N560 was made by subcloning the NdeI/EcoRI fragment of pCMV-HA-$hTAF_{II}250\Delta$N161 (see below) into pVL1392GST (Ruppert and Tjian, 1995). The construct pbGST-$hTAF_{II}250\Delta$N250/N560 was made by first inserting the XmnI (cDNA position 758)/EcoRI(cDNA position 1692) position into pGEX-2TK (Pharmacia) and then transferring the NdeI/EcoRI fragment derived thereof into pVL1392GST (Ruppert and Tjian, 1995).

Baculovirus Expression and Purification of HA- and GST-Tagged Recombinant Protein

Recombinant baculovirus was generated by cotransfection of the baculovirus transfer vectors and BaculoGold virus DNA (Pharmingen) into Sf9 cells (ATCC #CRL-1711) using lipofectin (GIBCO–BRL). After 4–5 days of incubation, the virus released into the medium was collected and used for amplification as described (O'Reilley et al., 1994). Extracts from infected cells were prepared as described (Ruppert et al., 1993). Crude extracts from cells expressing various HA- or GST-tagged wild-type and mutant $TAF_{II}250$ were bound to protein A sepharose beads coupled to anti-HA antibody or to GST beads (Pharmacia) in 0.5 M KCl–HEMG buffer for 2 or 0.5 hr, respectively. After 5 washes with the same buffer, the purified proteins were analyzed by SDS–PAGE. Before in vitro kinase assay, the beads were washed twice in kinase buffer.

In Vitro Kinase Assay and Phosphoamino Acid Analysis

Purification of TBP/TAF complexes was described previously (Dynlacht et al., 1991; Tanese et al., 1991). Immunopurified TFIID complex or tagged purified wild-type and mutants $TAF_{II}250$ proteins bound to the appropriate beads were incubated at 30°C for 30 min in 10–20 μl of kinase buffer containing 25 mM HEPES (pH 7.9), 12.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.1% NP-40, and 1–5 μCi [γ-^{32}P]ATP (5000 Ci/mmol; Amersham). The reactions were stopped by the addition of protein sample buffer followed by SDS–PAGE and autoradiography for 5–60 min. For phosphoamino acid analysis, phospho-proteins were transferred onto polyvinylidene difluoride membrane, excised, and hydrolyzed in 6 M guanidine-HCl at 110°C for 1 hr. Phosphoamino acids were separated on thin layer cellulose plates by electrophoresis in pH 3.5 buffer (glacial acetic acid:pyridine:water, 10:1:189) for 1 hr at 1500 V. The migration of cold phosphoamino acids was determined by ninhydrin staining, and the radioactive amino acids were visualized by autoradiography.

Denaturation/Renaturation Autophosphorylation Assay

This assay was carried out as described (Celenza and Carlson, 1986). In brief, the proteins to be analyzed (0.5–2 μg) were resolved on SDS–PAGE, transferred onto nitrocellulose membrane, and stained with ponceau S. After 2 washes with water, the immobilized proteins were denatured (7 M guanidine-HCl, 50 mM Tris (pH 7.9), 2 mM EDTA, and 10 mM dithiothreitol) for 1 hr at room temperature and allowed to renature for 12–16 hr at 4°C in kinase buffer. The membrane was then blocked with kinase buffer containing 5% nonfat dry milk for 30 min at room temperature and washed twice with kinase buffer followed by the addition of [γ-^{32}P]ATP to a final concentration of 100 μCi/ml for 30 min. The membrane was washed 5 times with 20 mM Tris (pH 7.5), 1% SDS and followed by autoradiography for 16–48 hr.

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