

Human TAF_{II}130 Is a Coactivator for NFATp

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NFATp is one member of a family of transcriptional activators that regulate the expression of cytokine genes. To study mechanisms of NFATp transcriptional activation, we established a reconstituted transcription system consisting of human components that is responsive to activation by full-length NFATp. The TATA-associated factor (TAF_{II}) subunits of the TFIID complex were required for NFATp-mediated activation in this transcription system, since TATA-binding protein (TBP) alone was insufficient in supporting activated transcription. In vitro interaction assays revealed that human TAF_{II}130 (hTAF_{II}130) and its *Drosophila melanogaster* homolog dTAF_{II}110 bound specifically and reproducibly to immobilized NFATp. Sequences contained in the C-terminal domain of NFATp (amino acids 688 to 921) were necessary and sufficient for hTAF_{II}130 binding. A partial TFIID complex assembled from recombinant hTBP, hTAF_{II}250, and hTAF_{II}130 supported NFATp-activated transcription, demonstrating the ability of hTAF_{II}130 to serve as a coactivator for NFATp in vitro. Overexpression of hTAF_{II}130 in Cos-1 cells inhibited NFATp activation of a luciferase reporter. These studies demonstrate that hTAF_{II}130 is a coactivator for NFATp and represent the first biochemical characterization of the mechanism of transcriptional activation by the NFAT family of activators.

Transcription is a highly regulated process of RNA synthesis, the initiation of which is a primary control point in gene expression. In eukaryotes, genes encoding mRNA are transcribed by RNA polymerase II, a multiprotein enzyme. Reconstitution of an in vitro transcription system with purified RNA polymerase II proved to be insufficient for RNA synthesis from promoters, propelling the identification of a phylogenetically conserved set of general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) that are necessary for basal transcription (reviewed in reference 45). The assembly of RNA polymerase II and the general transcription factors on promoter DNA leads to the formation of stable nucleoprotein complexes (preinitiation complexes) that are transcriptionally competent in vitro. Preinitiation complex formation can be regulated by the action of activators and repressors. The activities of these distinct regulatory proteins can promote (activators) or repress (repressors) the recruitment of general transcription factors and RNA polymerase II onto promoters, resulting in promoter-specific augmentation or suppression of transcription.

Eukaryotic organisms have evolved elaborate mechanisms for the deployment of RNA polymerase II onto specialized promoter elements that can modulate basal promoter strength. Many RNA polymerase II promoters contain one or more of three identified core promoter elements that direct accurate transcription initiation (5): the TATA box, the initiator element, and the downstream promoter element. In a temporal scheme, TFIID is the first basal factor to bind to a core promoter, serving as a nucleation center for the rest of the transcriptional apparatus (60). The architecture of TFIID is also

phylogenetically conserved, and it appears that TFIID complexes in yeast, *Drosophila melanogaster*, and humans have a similar complement of subunits (6). TFIID is a sequence-specific DNA binding general transcription factor consisting of TATA-binding protein (TBP) and 10 to 13 TATA-associated factors (TAF_{II}s) that together can recognize the TATA box, initiator, and downstream promoter elements.

The orchestration of transcriptional activation at mammalian promoters is a dynamic process that involves the recruitment of functional protein complexes in a highly regulated manner. Activators can exert their effects by interacting with components of the transcription machinery, including subunits of the TFIID and mediator complexes (19, 35), as well as by recruiting chromatin-modifying complexes (63). In general, it is the activation domains of activators that bind specific components of the transcriptional apparatus. Activation domains display target specificity depending on their amino acid composition and have been categorized as rich in a particular type of amino acid, for example, acidic residues, glutamines, or prolines (58).

A limiting step in the process of preinitiation complex formation at some promoters in vitro is the recruitment of TFIID, which is a common target of many eukaryotic activators (19). TFIID binding to promoters is a central event in transcription initiation, regardless of the manner by which the basal transcriptional components are assembled, either following a stepwise assembly or through the recruitment of a holoenzyme (7). In some cases, upstream activators that interact with TFIID components achieve efficient recruitment of TFIID (28, 54, 62). Although direct interactions between TBP and activators have been noted, the primary target within TFIID is not TBP but the TAF subunits of the TFIID complex. TAF_{II}s were coined coactivators in recognition of their requirement for activated transcription in in vitro systems (12, 48). The validity of the coactivator function of TAFs has been firmly established from examples in yeast, *Drosophila*, and human systems (1). Recently, the ability of TAF_{II}s to adopt multifunctional roles

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as components of functionally distinct complexes, including TFIID, SAGA, PCAF, and TFTC, has been demonstrated.

Although TAFs can function as coactivators, other mechanisms of transcriptional activation exist. The mediator complex, which was first identified in yeast and more recently in humans, is also a target of transcriptional activators and serves to coactivate transcription (35). In the context of chromatin, nucleosome remodeling complexes and histone acetyltransferases, such as CREB-binding protein (CBP), function to coactivate transcription (63). It is likely that activators can stimulate transcription by many different mechanisms depending upon the conditions and the context of the promoter at which the activator binds.

The nuclear factor of activated T cells (NFAT) family is a family of activators that exert a pivotal effect on the transcriptional regulation of genes encoding immunomodulatory cytokines (reviewed in reference 50). NFATp (NFAT1 or NFATc2) is one member of the NFAT family (39), which also includes NFATc (NFAT2 or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx), and NFAT5 (21, 37, 43). NFATp, NFATc, and NFAT4, which are activated during immune responses, participate in the activation of T cells (34, 50). A regulated burst of NFATc activity appears to be critical for proper cardiac muscle cell differentiation during embryogenesis (49). NFAT3 functions in cardiac hypertrophy (42). NFAT4 has been implicated in intrathymic development of immature thymocytes (37). NFAT5, also known as TonEBP, is involved in the transcriptional regulation of osmotic stress response genes (30, 41).

All NFAT proteins are DNA binding proteins whose DNA binding domains are conserved between family members and phylogenetically within mammalian systems (20, 21, 23, 30, 37). The DNA binding domains of NFAT proteins, with the exception of NFAT5, are sufficient for cooperative DNA binding in association with Fos-Jun heterodimers (21, 24, 30), and an X-ray structure revealed contacts between the DNA binding domain of NFAT and the basic leucine zipper domains of c-Fos and c-Jun (9). In contrast to the well-characterized DNA binding properties of NFAT proteins, less is known of the transactivation properties that are conferred by their activation domains. In the case of NFATp, two activation domains have been mapped to the N- and C-terminal regions flanking the central DNA binding domain using transient transfection assays and *in vitro* transcription experiments (25, 32). The N-terminal region of NFATp has been shown to interact with p300 and CBP and is proposed to play a role in transcriptional activation by NFATp in T cells (17). In general, the C-terminal regions of NFAT proteins exhibit great sequence variation, suggesting the possibility for functional differences between NFAT proteins.

NFAT transcriptional activity is regulated by cycles of dephosphorylation and phosphorylation involving the phosphatase calcineurin and a number of kinases (2, 3, 10, 11, 24, 29, 31, 36, 44, 47, 59, 65). A conserved regulatory domain located N terminal to the DNA binding domain of NFAT proteins has been shown to confer calcineurin-mediated nuclear localization (2, 33, 36). NFAT proteins exist as phosphoproteins in various tissues, but their activation in T cells has been investigated most thoroughly. Typically, antigen-stimulated T cells respond by activating a calcium-dependent signaling pathway

that promotes the activation of calcineurin (11). Activated calcineurin dephosphorylates NFAT proteins, which triggers the accumulation of NFAT proteins in nuclei (24, 43, 55). Treatment of cells with both a calcium ionophore and a phorbol ester mimics conditions conducive for NFAT nuclear accumulation and NFAT-activated transcription (14).

We discovered and characterized one mechanism by which NFATp activates transcription. Using a combination of protein-protein interaction assays, *in vitro* transcription experiments, and transient transfection assays we identified a target of NFATp in the general transcription machinery and investigated its role in mediating NFATp activation *in vitro* and in cells. Our results support a model in which the unique C-terminal region of NFATp binds the TAF_{II}130 subunit of human TFIID to activate transcription.

MATERIALS AND METHODS

Plasmids and baculoviruses. Plasmid pREP-NFATp was provided by Tim Hoey (Tularik, Inc.) (21). Plasmids pBS-KS⁺-NFATp, pVL1392-HAX-NFATp, pBS-KS⁺-NFATp(NdeI), and pVL-GST-NFATp have been described previously (25). pBS-NFATp(1-686) and pBS-NFATp(391-921) were generated using single-stranded pBS-KS⁺-NFATp(NdeI) for site-directed mutagenesis. In the case of pBS-NFATp(1-686) a stop codon was created in the NFATp cDNA after amino acid (aa) 686 using an oligonucleotide of sequence 5'-GACGGAGCCACGTAAGAATTCGACCCCACTCTG-3'. In the case of pBS-NFATp(391-921) a unique in-frame ATG codon contained in a *NdeI* site was created in place of the codon for aa 390 using an oligonucleotide of sequence 5'-CATCTGCAGCATCCATATGACTGCATCCCTC-3'. pTβ-NFATp(1-686) and pTβ-NFATp(391-921) were generated by subcloning *NdeI*-*EcoRI* fragments from pBS-NFATp(1-686) and pBS-NFATp(391-921), respectively, into plasmid pTβ-STOP (gift of R. Tjian) digested with *NdeI* and *EcoRI*. pGEX-NFATp(688-921) was created by using pBS-KS⁺-NFATp(NdeI) as template DNA in *NotI*-catalyzed (Promega) PCRs using primers PNF688-*NdeI* (5'-ATCTGGAATTCATATGGAATATGACCCCACTCTG-3') and PNF921-*EcoRI* (5'-ATCTGGAATTCCTCGGATCAAAGATCACAG-3'). This generated a DNA fragment that was digested with *NdeI* and *EcoRI* and subsequently ligated with *NdeI*- and *EcoRI*-cut pGEX-2TKN (gift of S. Ruppert and R. Tjian). Plasmids used for expression of TAFs have been described previously: pTβ-hTAF_{II}250 (52), pTβ-dTAF_{II}30α (64), pTβ-dTAF_{II}30β (64), pTβ-dTAF_{II}110 (22), and pTβ-dTAF_{II}150 (61) were provided by R. Tjian and pTβ-hTAF_{II}130, pTβ-hTAF_{II}100, and pCMV-hTAF_{II}130 were provided by N. Tanese (56).

pNFAT₃-MLP-G-less, used as template DNA in the *in vitro* transcription assays, consists of three direct copies of the murine interleukin-4 (IL-4) high-affinity NFAT site (region -82 to -64 of the murine IL-4 promoter) upstream of the adenovirus major late core promoter (-53 to +10) and a 200-bp G-less cassette. To create pNFAT₃-MLP-G-less a DNA fragment containing the three NFAT sites was recovered after digestion of pNFAT₃-E1B-CAT (25) with *XbaI* and was ligated into the *XbaI* site of pΔML200 (gift of J. Parvin) (4, 46). The reporter plasmid pGL3-NFAT₃-Luciferase was created by engineering *KpnI* and *SacI* sites into the ends of a PCR fragment bearing three NFAT sites from pNFAT₃-E1B-CAT. The PCR product was digested with *KpnI* and *SacI* and ligated into the pGL3-basic luciferase reporter construct (Promega).

pVL1392-HAX-NFATp and pVL-GST-NFATp were individually cotransfected with Baculogold DNA (PharmMingen) into Sf-9 insect cells to produce baculovirus stocks according to standard procedures. Baculoviruses used to express recombinant FLAG-hTAF_{II}100 and FLAG-hTAF_{II}130(137-1083) were a gift from N. Tanese. The HA-hTAF_{II}250 baculovirus was a gift from R. Tjian.

Expression and purification of recombinant NFATp proteins. Recombinant hemagglutinin (HA)-NFATp was purified as previously described (25). For the preparation of HA-NFATp, Hi-five cells were infected with the HA-NFATp virus for 44 h at 27°C. Cells were harvested by low-speed centrifugation and resuspended in lysis buffer (20 mM Tris [pH 7.9], 20% [vol/vol] glycerol, 1 mM EDTA, 0.5 M NaCl, 0.1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg of leupeptin/ml, 1.4 μg of pepstatin A/ml, and 1 mM dithiothreitol [DTT]). Sonication was performed at 4°C for 5 min before centrifugation at 16,000 × g for 20 to 30 min. The recombinant protein was purified on anti-HA-conjugated beads and washed two times in TGEMD buffer (20 mM Tris [pH 7.9], 20% [vol/vol] glycerol, 1 mM EDTA, 5 mM MgCl₂, 0.1% NP-40, 0.2 mM PMSF, 1 μg of leupeptin/ml, 1.4 μg of pepstatin A/ml, and 1 mM DTT) containing 1 M

NaCl and an additional two times in TGEMD buffer containing 0.2 M NaCl. HA-NFATp was subsequently eluted with 1 mg of HA peptide/ml in elution buffer (20 mM Tris [pH 7.9], 20% [vol/vol] glycerol, 1 mM EDTA, 5 mM MgCl₂, 0.2 M NaCl, 0.1% NP-40, 0.2 mM PMSF, 1 µg of leupeptin/ml, 1.4 µg of pepstatin A/ml, and 1 mM DTT). The eluate was spun through a Millex-GV4 filter, frozen in liquid nitrogen, and stored at -80°C. Glutathione *S*-transferase (GST) NFATp (in Hi-five cells), FLAG-hTAF_{II}130 (in Sf-9 cells), FLAG-hTAF_{II}100 (in Sf-9 cells), and HA-hTAF_{II}250 (in Sf-9 cells) were expressed in the indicated cells using a similar protocol, with the exception that cell lysates were aliquoted and frozen prior to purification on the appropriate affinity resin (see below).

GST-NFATp(688-921) was expressed in *Escherichia coli* strain XA-90. Cultures transformed with pGEX-NFATp(688-921) were grown in the presence of 100 µg of ampicillin/ml in Luria-Bertani broth at 37°C to an optical density of 0.4 at 600 nm before expression was induced by the addition of isopropylthio-β-D-galactoside at a 0.6 mM final concentration. After 2 h, cells were harvested and the cell pellet was resuspended in a buffer containing 20 mM Tris (pH 7.9), 20% (vol/vol) glycerol, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.2 mM PMSF and sonicated with four 20-s pulses. After centrifugation, the supernatant was aliquoted, frozen in liquid nitrogen, and stored at -80°C.

Protein-protein interaction assays. GST fusion proteins and GST were immobilized by incubating cell extracts with glutathione-Sepharose beads for 2 h at 4°C. Immobilized proteins were washed two times in 10-bead volumes of TGEMD buffer containing 1 M NaCl [TGEMD (1 M NaCl)] followed by two washes in 10-bead volumes of TGEMD buffer containing 0.1 M NaCl [TGEMD (0.1 M NaCl)]. Prior to use in interaction assays, the amount of each protein on the beads was estimated and approximately 0.5 µg of total immobilized protein was added to each interaction assay. Input proteins either were made in a rabbit reticulocyte transcription-translation system (TNT; Promega) in the presence of [³⁵S]methionine, were provided by insect cell lysates containing FLAG-hTAF_{II}130 (see Fig. 3C), or consisted of purified TFIID and TBP (see Fig. 3D). Immobilized proteins and extracts containing target (input) proteins were incubated with micrococcal nuclease at 30°C for 10 min. Input proteins were added to the immobilized GST fusion proteins and nutated for 2 h at 4°C. After washing four times with 10-bead volumes of TGEMD (0.1 M NaCl), the bound fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (or phosphorimager), silver staining, or immunoblotting (as indicated in the figure legends). Similar conditions were used for interaction assays with immunoprecipitated FLAG-hTAF_{II}130 and NFATp, except that the FLAG-hTAF_{II}130 was purified on M2 affinity beads (Sigma).

Assembly of partial TFIID complexes. Partial TFIID complexes, hTBP-hTAF_{II}250-hTAF_{II}130 and hTBP-hTAF_{II}250-hTAF_{II}100, were assembled in multiple steps (see Fig. 4A). In summary, the HA-TAF_{II}250-hTBP complex was preassembled and eluted prior to its addition to the second binding reaction with immobilized FLAG-hTAF_{II}130 or FLAG-hTAF_{II}100. First, HA-hTAF_{II}250 was purified from an Sf-9 extract by nutation with anti-HA antibody immobilized on protein A-Sepharose 4 Fast Flow (Amersham Pharmacia) for 6 h at 4°C. Immobilized HA-hTAF_{II}250 was washed extensively with TGEMD (1 M NaCl) followed by TGEMD (0.2 M NaCl). Second, a partially purified fraction of human TBP (hTBP) (expressed in *E. coli*) was added to the immobilized HA-hTAF_{II}250 and nutated overnight at 4°C. Third, FLAG-hTAF_{II}130 and FLAG-hTAF_{II}100 were purified from insect cell extracts by nutation with anti-FLAG M2 affinity beads for 4 h at 4°C. Non-specifically bound proteins were removed by a series of washes in TGEMD (1.0 M NaCl), followed by TGEMD (0.1 M NaCl). Fourth, after washing in TGEMD (0.2 M NaCl) the HA-TAF_{II}250-TBP complex was eluted in a 2-h nutation with 1 µg of HA peptide/µl that was resuspended in elution buffer (20 mM Tris [pH 7.9], 20% glycerol, 1 mM EDTA, 5 mM MgCl₂, 0.2 M NaCl, 0.1% NP-40, 2 mM PMSF, 1 mM DTT, 1 µg of leupeptin/ml, 1.4 µg of pepstatin A/ml). Fifth, the eluted HA-TAF_{II}250-TBP complex was added to immobilized FLAG-hTAF_{II}130 and FLAG-hTAF_{II}100 and nutated for 6 h at 4°C. Sixth, the beads were washed four times in TGEMD (0.1 M NaCl). Finally, FLAG peptide (Sigma) was used at a final concentration of 0.5 mg/ml in elution buffer to elute the complexes during a 2-h nutation at 4°C. The eluted fractions were spun through a Millex-GV4 filter, frozen in liquid nitrogen, and stored at -80°C.

In vitro transcription. Transcription reactions using the reconstituted transcription system were performed as previously described (16), with the following modifications. Each reaction contained 50 ng of NFAT₃-MLP-G-less template. Where indicated, HA-NFATp (50 ng) was preincubated with promoter DNA for 5 min at 30°C. TFIID, TBP, or partial TFIID complexes were added to reactions at equivalent final molar concentrations (as determined by anti-TBP immunoblotting; 16) and incubated for 15 min at 30°C prior to the addition of the remaining general transcription factors (TFIIA, TFIIB, TFIIE, TFIIF, and

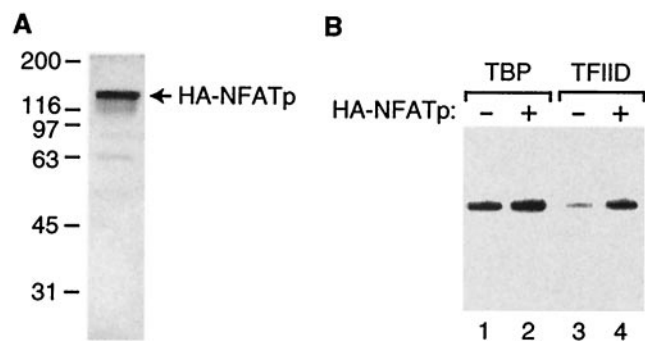


FIG. 1. TAF subunits of TFIID act as coactivators for NFATp transcriptional activation in vitro. (A) Purification of recombinant HA-tagged human NFATp. HA-NFATp was expressed in insect cells using a recombinant baculovirus. The protein was purified from cell extracts by anti-HA affinity chromatography and eluted with an HA-epitope peptide. Approximately 200 ng of recombinant protein was resolved by SDS-PAGE and analyzed by silver staining. (B) NFATp transcriptional activation in a reconstituted human transcription system is dependent on the TAF subunits of TFIID. Transcription reactions were reconstituted with purified human general transcription factors (TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH) and RNA polymerase II. Reactions contained equivalent molar concentrations of either immunopurified human TFIID (consisting of TBP and TAFs; lanes 3 and 4) or recombinant human TBP (lanes 1 and 2). NFATp was included in reactions as indicated. The DNA template contained three NFAT sites upstream of the adenovirus major late core promoter and a 200-bp G-less cassette. RNA products were recovered and analyzed by denaturing PAGE. The resulting gel was dried and subjected to autoradiography.

TFIIH) and RNA polymerase II. After the addition of the general transcription factors, reactions were incubated at 30°C for 20 min. Nucleoside triphosphates were added and RNA synthesis was allowed to proceed for 20 min at 30°C. Transcription reactions were stopped with 100 µl of a solution containing 3.1 M ammonium acetate, 10 µg of carrier yeast RNA, and 15 µg of proteinase K. After ethanol precipitation, the samples were resolved by 6% denaturing PAGE and analyzed with a PhosphorImager.

Transfection assays. Cos-1 cells were transfected with Lipofectamine reagent (GIBCO) for 6 h at 37°C using 1.3 µg of total DNA and 3.2 µl of Lipofectamine. Transfections consisted of the following quantities of DNA (see legend to Fig. 5 for amounts used in specific experiments): 50 to 500 ng of pREP4-RSV-NFATp or the parental vector, 100 ng of pCMV-hTAF_{II}130 or the pCMV parental vector, 1 µg of pGL-NFAT₃-Luc, and 100 ng of pRL-TK-Renilla luciferase reporter. Where indicated, cells were stimulated with 1 µM ionomycin (Calbiochem) and 20 µg of phorbol-12-myristate-13-acetate (PMA)/ml for various times at 37°C prior to harvest. Cell lysates were prepared by sonication for 5 min at 4°C in 1× lysis buffer (Promega). The dual luciferase reporter assay system (Promega), which includes the analysis of firefly luciferase and Renilla luciferase within a single sample, was used for the quantification of reporter gene expression. Luciferase activities were determined using an EG&G Berthold's Microlum LB96P luminometer. NFAT-driven firefly luciferase activity was normalized by dividing firefly luciferase units by the units corresponding to Renilla luciferase activity.

RESULTS

To study mechanisms of transcriptional activation by human NFATp we developed a protocol for the expression and purification of recombinant human NFATp containing an N-terminal HA tag in insect cells using a recombinant baculovirus (25). HA-NFATp was purified from insect cell lysates using anti-HA affinity chromatography followed by elution with an epitope peptide. The purification resulted in a nearly homogeneous preparation of HA-NFATp (Fig. 1A). The recombinant protein was functional in DNA binding on its own as well as in

cooperation with AP-1 proteins, as assessed by DNase I footprinting (25).

TFIID TAFs function as coactivators for NFATp transcriptional activation in a reconstituted transcription system. Analysis of the transcriptional properties unique to NFATp required the creation of an NFAT-responsive promoter for in vitro transcription studies and the development of a human reconstituted transcription system that is responsive to NFATp. In designing an NFATp-responsive DNA template, we positioned three high-affinity NFAT-binding sites upstream of the adenovirus major late core promoter and a 200-bp G-less cassette. For in vitro transcription studies we utilized a system consisting of purified recombinant and native human general transcription factors, including recombinant TFIIA (rTFIIA), rTFIIB, rTFIIE, rTFIIF, native TFIID (nTFIID), nRNA polymerase II, and either nTFIID or rTBP. We previously showed that this transcription system is responsive to recombinant NFATp, that NFATp activation using the reporter template is not dependent on AP-1 proteins, and that AP-1 proteins do not contaminate the purified transcription system (25). The reconstituted transcription system provided us with a tool to study mechanisms of transcriptional activation by NFATp in the absence of AP-1 proteins and allowed us to investigate the roles of TFIID TAFs in NFATp activation.

HA-NFATp activated transcription an average of sevenfold above the basal level in the reconstituted transcription system containing holo-TFIID (Fig. 1B, compare lanes 3 and 4). Reactions reconstituted with TBP in place of TFIID showed at most a 50% increase over basal transcription (Fig. 1B, lanes 1 and 2) and on average a 20% increase over basal transcription (i.e., 1.2-fold activation). Hence, the TAF subunits of TFIID were required for high levels of transcriptional activation by HA-NFATp. In this transcription system we observe a higher level of basal transcription when using TBP in place of TFIID even though equivalent molar concentrations of TBP and TFIID were used (16). This is likely to be due to domains present in the TFIID complex that inhibit its binding to DNA, such as the N terminus of TAF_{II}250 (27). Titration of TBP to lower levels did not allow a higher level of transcriptional activation by NFATp (data not shown). Control reactions that lacked either TBP or TFIID but were complemented with the activator and all other components of the basal transcription machinery showed no detectable RNA synthesis (data not shown; see Fig. 4D), indicating that the transcription system is absolutely dependent on an external source of TBP or TFIID. Consistent with current models of transcriptional activation by many mammalian activators, our experiments demonstrate that transcriptional activation by NFATp can be mediated by the TAF subunits of TFIID in vitro.

NFATp binds hTAF_{II}130 and dTAF_{II}110. It has previously been observed that activation domains display binding specificity toward distinct TAF subunits of the TFIID complex (19). We tested NFATp for the ability to interact with TAFs using in vitro protein-protein interaction assays. To perform the interaction assays, we expressed NFATp as a GST fusion protein in insect cells using a recombinant baculovirus. Purified GST-NFATp bound DNA and activated transcription in vitro (25). As a convenient source of TAFs for the initial round of interaction assays we expressed a variety of *Drosophila* and human TAFs using in vitro transcription and translation in rabbit

reticulocyte lysate. GST-NFATp was immobilized on glutathione-Sepharose beads, mixed with input TAF proteins for several hours, and washed extensively, and bound protein was analyzed by SDS-PAGE and autoradiography. GST-NFATp interacted specifically with *Drosophila* TAF_{II}110 (dTAF_{II}110) but not with dTAF_{II}30 α , dTAF_{II}30 β , dTAF_{II}150, or human TAF_{II}250 (hTAF_{II}250) (Fig. 2A). In all cases, control GST protein that was immobilized failed to interact with input proteins or interacted less well than immobilized GST-NFATp. To further examine the interaction between GST-NFATp and TAFs we obtained expression vectors for two other human TAFs, hTAF_{II}130 (the homolog of dTAF_{II}110) and hTAF_{II}100 (the homolog of dTAF_{II}80). As shown in Fig. 2B, GST-NFATp interacted with hTAF_{II}130 but not with hTAF_{II}100. As an additional test of the interaction between NFATp and hTAF_{II}130 we immobilized FLAG-hTAF_{II}130 that was expressed in insect cells on M2 affinity beads. The immobilized FLAG-hTAF_{II}130 was incubated with HA-NFATp (purified from insect cell lysates). After extensive washing, bound protein was resolved by SDS-PAGE and analyzed by silver staining. As shown in Fig. 2C, NFATp bound immobilized hTAF_{II}130. Together these results demonstrate that the human and *Drosophila* homologs of a subunit of TFIID interact with NFATp.

The C-terminal transactivation domain of NFATp is necessary and sufficient for hTAF_{II}130 interaction. To identify the region of NFATp that interacts with hTAF_{II}130, in vitro protein-protein interaction assays were conducted using deletion mutants representing N- and C-terminal truncations of NFATp. NFATp(1-686) contains the N-terminal transactivation domain, the calcineurin binding domain, and the DNA binding domain (Fig. 3A). NFATp(391-921) contains the DNA binding domain and the C-terminal transactivation domain. Along with the full-length version of NFATp, these deletion mutants were in vitro translated in the presence of [³⁵S]methionine. FLAG-hTAF_{II}130 expressed in insect cells was immobilized on M2 affinity beads, in parallel with control M2 beads that were exposed to an insect cell extract lacking expressed FLAG-hTAF_{II}130. Full-length and truncated NFATp were added to these beads, and after sufficient mixing, reactions were subjected to a series of washes before analysis by SDS-PAGE and autoradiography (Fig. 3B). Full-length NFATp and NFATp(391-921) bound to immobilized FLAG-hTAF_{II}130. In contrast, NFATp(1-686) did not interact with hTAF_{II}130. Binding was dependent on the presence of hTAF_{II}130, since control beads that were not exposed to recombinant hTAF_{II}130 failed to bind NFATp. Our results show that the C-terminal region of NFATp (aa 687 to 921) is required for interaction with hTAF_{II}130.

Next, we asked if the C-terminal region of NFATp is sufficient to support protein-protein interaction if separated from the rest of the NFATp protein. To address this issue, we expressed and purified GST-NFATp(688-921). GST-NFATp(688-921) and control GST were separately expressed in *E. coli*, and both were immobilized onto glutathione-Sepharose beads. As a positive control, full-length GST-NFATp and its GST control made in insect cells were tested in parallel reactions. Recombinant hTAF_{II}130 purified from insect extract was incubated with the immobilized proteins. Proteins that bound to the beads were separated by SDS-PAGE and analyzed by protein immunoblotting with a monoclonal antibody

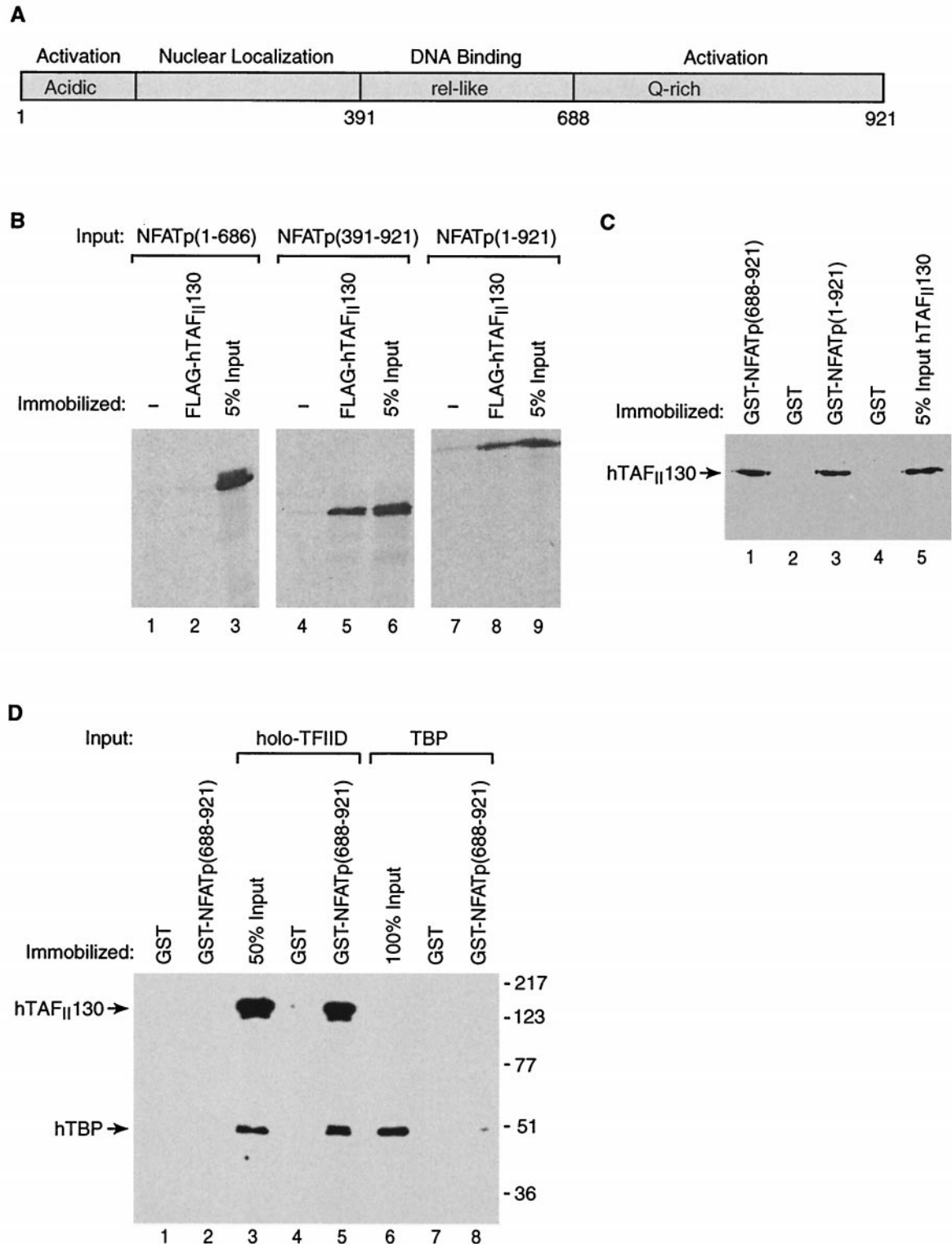


FIG. 3. The C-terminal region of NFATp is both necessary and sufficient for interaction with hTAF_{II}130. (A) Schematic of the functional domains of NFATp. The DNA binding domain is central to the protein and located between aa 391 and 688. The N-terminal region (aa 1 to 390) contains an activation domain rich in acidic amino acids and the region of the protein that binds calcineurin and is involved in regulated nuclear localization. The C-terminal region (aa 688 to 921) is unique to NFATp and contains an activation domain that is rich in glutamines. (B) The C-terminal region of NFATp is required for interaction with hTAF_{II}130. Extracts from Hi-five cells in which FLAG-hTAF_{II}130 had been expressed and control extracts were incubated with M2 affinity beads. Full-length NFATp and two deletions of NFATp that were produced by *in vitro* transcription and translation in the presence of [³⁵S] methionine were incubated with immobilized FLAG-hTAF_{II}130 and control beads. After washing away unbound protein, bound protein was analyzed by SDS-PAGE and autoradiography. (C) The C-terminal region of NFATp is sufficient for hTAF_{II}130 interaction. Bacterially expressed GST-NFATp(688-921), Hi-five-expressed GST-NFATp(1-921), and control GST proteins

staining indicated that individual subunits were incorporated at comparable levels into the partial TFIID complexes (Fig. 4C).

Using the reconstituted transcription system described earlier, we compared the ability of NFATp to activate transcription in reactions reconstituted with either TFIID, the hTBP/hTAF_{II}250/hTAF_{II}130 complex, or the hTBP-hTAF_{II}250-hTAF_{II}100 complex (Fig. 4D). Consistent with the results of the interaction assays, the hTBP-hTAF_{II}250-hTAF_{II}130 complex supported an average of sixfold transcriptional activation by NFATp (compare lanes 5 and 6). In contrast, reactions reconstituted with the partial TFIID complex containing hTAF_{II}100 in place of hTAF_{II}130 were not able to support significant NFATp-activated transcription (average activation of 1.2-fold; compare lanes 3 and 4), consistent with the lack of observed interaction between NFATp and either hTAF_{II}100 or hTAF_{II}250. These results demonstrate that hTAF_{II}130 can act as a coactivator for NFATp *in vitro*.

Overexpression of hTAF_{II}130 inhibits NFATp-activated transcription in cells. In previous studies, transient overexpression of hTAF_{II}130 in cells had differing effects on transcriptional activation. The exogenous expression of hTAF_{II}130 potentiated the level of activation by some activators (40) while repressing or having a dominant-negative effect on activation by other activators (38, 53). To conduct similar experiments, we constructed an NFAT-responsive reporter by introducing three NFAT sites upstream of the E1B-TATA core promoter and the firefly luciferase gene. We tested this reporter for responsiveness to overexpressed NFATp in Cos-1 cells. Transfection efficiency was monitored in all samples by cotransfection with a pRT-TK-*Renilla* luciferase plasmid. As expected, increasing amounts of NFATp induced firefly luciferase expression from the NFAT-dependent reporter; however, a control template lacking NFAT sites failed to respond (data not shown).

To assess the effect of hTAF_{II}130 overexpression on NFAT-directed transcriptional activation, Cos-1 cells were cotransfected with expression plasmids for NFATp and hTAF_{II}130. Portions of the transfected cell cultures were stimulated with ionomycin and PMA for various amounts of time (6, 12, and 24 h) or were unstimulated. Localization of NFATp is not dependent on ionomycin treatment in Cos cells (31); however, it was applied to cells to simulate the physiological conditions that may be conducive to NFAT-mediated activation. Similarly, cells were treated with PMA to activate PMA-responsive effectors that may support NFAT-mediated activation *in vivo*. Firefly luciferase values were normalized by *Renilla* luciferase values for each sample. As shown in Fig. 5A, NFATp caused a 6- to 10-fold increase in luciferase activity that did not depend on treatment with PMA and ionomycin. Expression of hTAF_{II}130 did not affect luciferase activity in the absence of NFATp overexpression; however, expression of hTAF_{II}130

significantly decreased NFATp-mediated luciferase activity under all conditions tested. The amount of hTAF_{II}130 expression vector added to the transfections did not decrease the expression level of *Renilla* luciferase, which is driven by the thymidine kinase promoter (data not shown). Furthermore, the repressive effect of hTAF_{II}130 overexpression on NFATp activation was overcome by increasing the amount of NFATp expression plasmid added to transfections (Fig. 5B). We conclude that overexpressed hTAF_{II}130 acts as a dominant-negative inhibitor of NFATp-activated transcription in cells.

DISCUSSION

Here we present evidence supporting a mechanism by which NFATp potentiates transcriptional activation by direct interaction with hTAF_{II}130, a component of the TFIID complex. Such targeting events by activators to their TAF coactivator partners are thought to enhance the recruitment and/or stabilization of TFIID to promoters. Our model of a mechanism of NFAT-mediated activation is strongly supported by three key results: (i) the presence of a direct and specific interaction between NFATp and hTAF_{II}130 *in vitro*, (ii) the ability of hTAF_{II}130 to serve as a coactivator for NFATp *in vitro*, and (iii) the observation that overexpressed hTAF_{II}130 repressed NFATp activation in cells. This study is the first to demonstrate a mechanism of activation by any NFAT family member using a cell-free system.

The C-terminal domain of NFATp (aa 688 to 921) is both necessary and sufficient to bind hTAF_{II}130. This region contains a glutamine-rich activation domain. Since hTAF_{II}130 and dTAF_{II}110 have been found to bind Gln-rich activation domains of other activators such as CREB and SP-1 (13, 22, 53, 56), Gln-rich regions of the C-terminal domain of NFATp may be important in hTAF_{II}130-mediated recruitment of TFIID. Human NFATp exists as predominately two isoforms (B and C) that are splice variants, with differences only in the extreme tail end of these proteins (aa 908 to 921 and 908 to 925, respectively) (50). In the studies described here we used the B isoform of NFATp. It remains to be determined if the hTAF_{II}130-dependent mode of activation characterized here is also applicable to the C isoform of NFATp. In either case, the amino acids shared in the C-terminal regions of the B and C isoforms of NFATp (aa 688 to 908) are unique to NFATp and are not found in other NFAT proteins, and hence it is possible that interaction with hTAF_{II}130 is a unique property of NFATp. Future studies will be aimed at testing for TAF interactions with other NFAT family members.

When hTAF_{II}130 was overexpressed in cells, we observed a dominant-negative effect on NFAT-activated transcription. This phenomenon has previously been observed for a number of viral and cellular activators in mammalian cells. Transcrip-

expressed in bacteria (lane 2) and Hi-five cells (lane 4) were immobilized on glutathione-Sepharose beads. Recombinant FLAG-hTAF_{II}130 purified from Sf-9 extracts was incubated with the immobilized GST fusion proteins. After extensive washing, bound protein was analyzed by immunoblotting with anti-hTAF_{II}130. (D) Holo-TFIID, but not TBP, binds the C-terminal domain of NFATp. Equivalent molar amounts of purified holo-TFIID and recombinant human TBP were incubated with immobilized GST-NFATp(688-921) and GST. After extensive washing, bound protein was resolved by SDS-PAGE and visualized by immunoblotting with anti-hTAF_{II}130 and anti-TBP antibodies. Portions of the input TFIID and TBP were included in lanes 3 and 6. Controls for immunoreactivity of the GST and GST-NFATp(688-921) were included in lanes 1 and 2. The positions of hTAF_{II}130 and TBP are indicated.

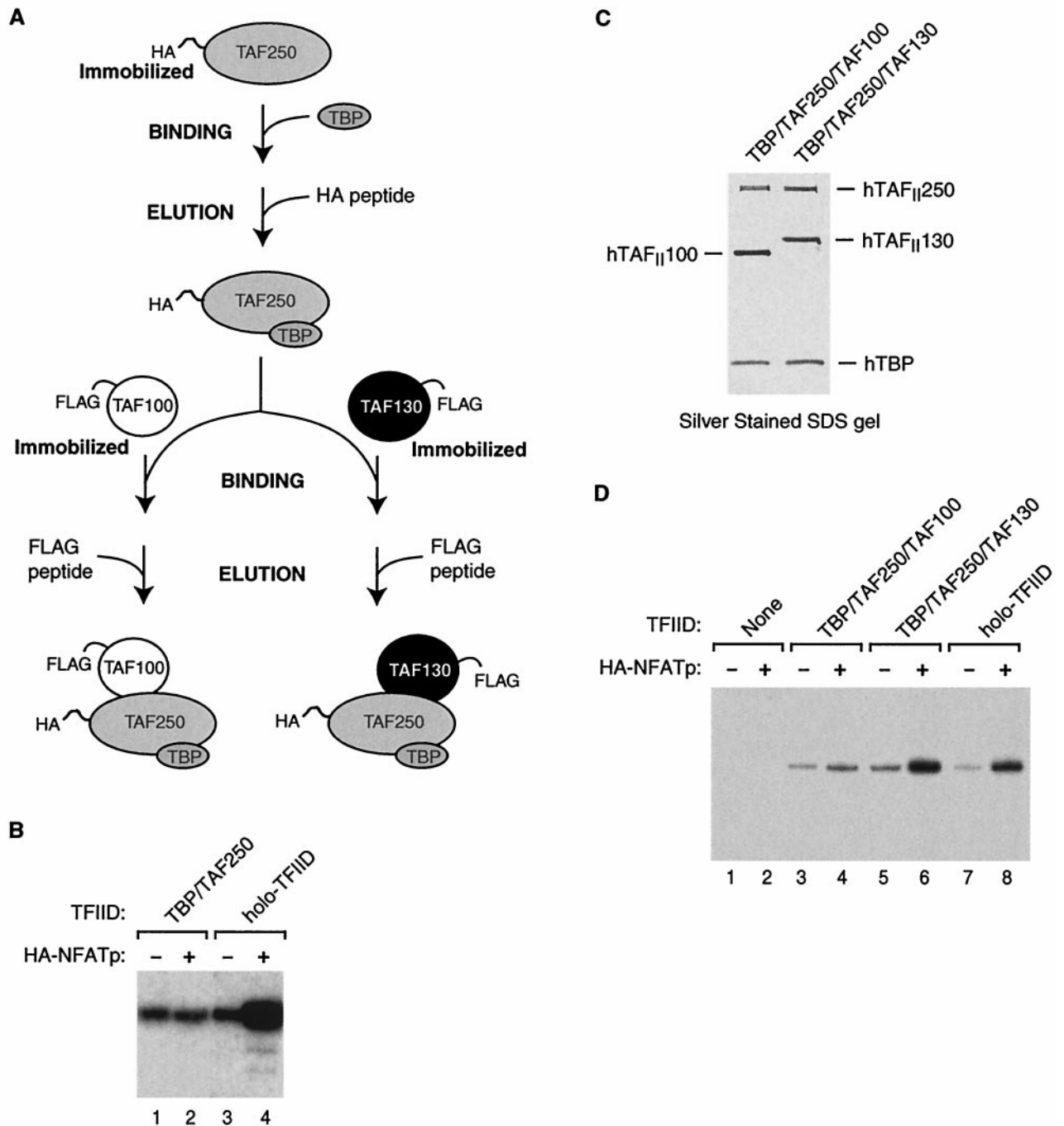


FIG. 4. hTAF_{II}130 is a coactivator for NFATp transcriptional activation in vitro. (A) Schematic depicting the method used for the assembly of the partial TFIID complexes that were tested for the ability to support NFATp activation in vitro. Two types of partial TFIID complexes were assembled that differed in composition by a single component, the incorporation of either hTAF_{II}130 or hTAF_{II}100. The assembly was performed in a step-wise manner. (i) Recombinant HA-hTAF_{II}250 was bound to anti-HA affinity beads; (ii) recombinant TBP was incubated with immobilized HA-hTAF_{II}250; (iii) the HA-hTAF_{II}250/TBP complex was eluted with an HA-epitope peptide; (iv) recombinant FLAG-hTAF_{II}130 and FLAG-hTAF_{II}100 were separately immobilized on M2 beads; (v) preassembled HA-hTAF_{II}250-TBP complex was incubated with both immobilized FLAG-hTAF_{II}130 and immobilized FLAG-hTAF_{II}100; and (vi) ternary complexes were eluted with the FLAG-epitope peptide. (B) The hTAF_{II}250-TBP complex supports basal but not NFATp-activated transcription. The HA-TAF_{II}250-TBP complex was substituted for immunopurified TFIID in a reconstituted transcription assay in the absence and presence of NFATp. RNA products were purified, resolved by denaturing PAGE, and subjected to PhosphorImager analysis. (C) Silver-stained SDS gel showing the two assembled partial TFIID complexes. The positions of the subunits are indicated. (D) The partial complex containing hTAF_{II}130, but not the partial complex containing hTAF_{II}100, can replace holo-TFIID in mediating NFATp transcriptional activation in vitro. The two partial TFIID complexes, TBP-TAF_{II}250-TAF_{II}130 and TBP-TAF_{II}250-TAF_{II}100, were substituted for immunopurified TFIID in a reconstituted transcription assay in the absence and presence of NFATp. RNA products were purified, resolved by denaturing PAGE, and subjected to PhosphorImager analysis.

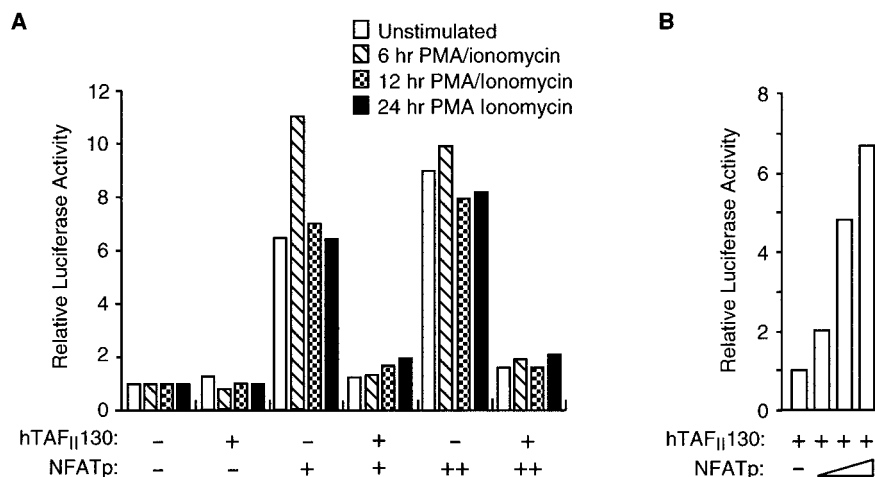


FIG. 5. Overexpression of hTAF_{II}130 inhibits NFATp-activated transcription in cells. (A) Cos-1 cells were transfected with plasmids encoding NFATp (50 or 100 ng) and hTAF_{II}130 (100 ng) as indicated. An NFAT reporter template consisting of three NFAT sites upstream of the E1B TATA box and the firefly luciferase gene and a control *Renilla* luciferase reporter were included in all transfections. The effects of NFATp and hTAF_{II}130 were assayed by measuring the luciferase activities of extracts prepared from cells harvested without stimulation or at three time points of stimulation with PMA and ionomycin (6, 12, and 24 h). For each sample, the firefly luciferase reporter activity was normalized by its respective *Renilla* luciferase activity. Normalized luciferase activities were plotted relative to activities present in samples from cells transfected with neither the NFATp- nor TAF_{II}130-expressing plasmids. (B) Increased levels of NFATp expression vector overcome the hTAF_{II}130 repression of NFATp activation. Transfection assays were performed as described above except that the amounts of NFATp expression vector used were 0, 100, 300, and 500 ng. Cells were stimulated with PMA and ionomycin for 6 h.

tional activation by SP-1 and CREB, which can individually associate with hTAF_{II}130 and dTAF_{II}110 in vitro, was inhibited by overexpression of hTAF_{II}130 in mammalian cells (53). Similarly, it has been shown that E1A binds directly to hTAF_{II}130 in vitro and that overexpression of the hTAF_{II}130 C-terminal domain repressed E1A-mediated transcriptional activation in cells in a dosage-dependent manner (18, 38). The authors of these studies have offered the following explanations for the dominant-negative effect of hTAF_{II}130 overexpression that can also be applied to the NFATp system. It is possible to envision promoter-bound activators preferentially binding the more abundant overexpressed hTAF_{II}130 instead of recruiting the entire TFIID complex. This would result in the loss of transcriptional activation. It is equally conceivable that hTAF_{II}130 overexpression causes a diluting effect of another essential factor that functions in hTAF_{II}130-mediated transcriptional activation by these activators. Our observations support the first explanation provided above in that the repressive effect of hTAF_{II}130 overexpression on NFATp activation was overcome by increasing the amount of NFATp expression plasmid added to transfections.

Promoters, such as the IL-2 promoter, that are regulated by NFAT often contain multiple NFAT sites, many of which are part of composite elements to which NFAT and AP-1 bind cooperatively (51). It is likely that NFATp affects transcription at many levels in cells by targeting the TAF_{II}130 subunit of the TFIID complex as well as non-TFIID factors. Our results suggest that if additional coactivators for NFATp exist in cells, they are factors other than the general transcription factors or the RNA polymerase II subunits, since TBP-supported transcription reactions were nonresponsive to NFATp. For example, the coactivators p300 and CBP have been found to bind NFATp (17).

Here we have shown that NFATp binds the TAF_{II}130 subunit of human TFIID and that hTAF_{II}130 is a coactivator for NFATp in a reconstituted transcription system. These in vitro studies provide the first biochemical analysis of mechanisms of transcriptional activation by the NFAT family of transactivators. We also found that overexpressed hTAF_{II}130 blocked NFATp-activated transcription in cells. It seems likely that the interaction between NFATp and hTAF_{II}130 plays a role in transcriptional activation at endogenous promoters; however, the identity of these promoters and the exact role of this interaction in the temporal and cell-type-specific expression of NFATp-responsive promoters awaits further study.

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