Isolation and Characterization of TAF25, an Essential Yeast Gene That Encodes an RNA Polymerase II-specific TATA-binding Protein-associated Factor*

(Received for publication, September 18, 1995, and in revised form, November 29, 1995)

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We describe the cloning and analysis of TAF25, a previously uncharacterized yeast gene that encodes a yeast TATA-binding protein-associated factor or yTAF of $M_r = 25,000$. The gene encoding yTAF25 is a single copy essential gene, and the protein sequence deduced from TAF25 exhibits sequence similarity to a metazoan hTAF11. The results from immunological studies confirm that yTAF25 is a subunit of a large multiprotein TATA-binding protein (TBP)-associated factor complex that contains a subset of the total number of the yTAFs present in yeast cell extracts. Both genetic and biochemical analyses demonstrate that yTAF25 can interact directly with itself. Transcriptional data show that the activity of the multiprotein complex containing yTAF25 is RNA polymerase II-specific, thus indicating that TAF25 encodes a bona fide yeast RNA polymerase II TAF. Hence the protein encoded by TAF25 has been termed yTAF11.

The TATA-binding protein (TBP)* is required for transcription by all three eukaryotic nuclear DNA-dependent RNA polymerases (Refs. 1–4; reviewed in Refs. 5 and 6), where it plays an essential but as of yet incompletely understood role in transcription. In all the systems studied thus far (Drosophila, human, and yeast) TBP exists as a component of multisubunit complexes comprised of TBP and TBP-associated factors (TAFs) (1, 7–12), (see Ref. 13 and 14 for recent reviews). The paradigm that has emerged from these studies is that the polymerase specificity of a particular TBP-TAF complex is determined by the collection of TAFs associated with TBP and not by the TBP molecule itself. Attempts are now being made to define the total number of TAFs, the number and composition of discrete TBP-TAF complexes, and the minimal TBP-TAF complements for each RNA polymerase. Through these efforts several distinct TBP-TAF complexes have been identified and characterized. The RNA polymerase I-specific TBP-TAF complex termed SL1 contains three TBP-associated proteins or TAF$_1$s (1, 15, 16), whereas the RNA polymerase II-specific TBP-TAF complex termed TFIIID contains eight to twelve TAF$_1$s (10, 17–20). Similarly, the RNA polymerase III TBP-TAF complex, TFIIIB, contains two TAF$_1$s (21–23) at least in yeast. A fourth TBP-TAF complex, termed the SNAP complex or SNAP$_c$, appears to be involved in directing small nuclear RNA gene transcription by RNA polymerases II and III and consists of at least three TAFs (24). The various TBP-TAF complexes (SL1, TFIIID, TFIIIB, and SNAP$_c$) subserve key regulatory functions in the transcription process at the level of transcription initiation. In addition to these initiation-competent TBP-TAF complexes, there are additional TAFs that appear to negatively modulate transcription, perhaps by sequestering TBP thereby affecting its ability to form initiation competent TBP-TAF complexes (25–28).

In recent years our studies have been directed toward defining TBP-TAF complexes in the yeast Saccharomyces cerevisiae. We previously reported the use of anti-TBP antibodies for immunofluorescence immunoblotography to purify TBP-TAF complexes from yeast whole cell extracts (WCE) (29). The TBP-TAF complexes in this TAF fraction reproducibly contains at least nine polypeptides ranging in size from $M_r = 170,000$ to $M_r = 25,000$. In the process of characterizing the proteins in this TAF fraction, we have determined that these TAFs are derived from at least three distinct TBP-TAF complexes. The transcription factor TFIIIB, a TBP-TAF complex specific to the function of RNA polymerase III, was the first complex we identified in this fraction (29). Yeast TFIIIB has been shown to contain TBP and two yTAF$_1$s, an $M_r = 70,000$ subunit termed B′ (or, alternatively, Brf1p, Tds4p, or Pcf4p) (30–32) and an $M_r = 90,000$ subunit termed B″ (22, 33). Both B′ (Brf1p) and B″ are present in our yTAF preparation as demonstrated by the fact that we can reconstitute specific mRNA gene transcription in vitro using purified RNA polymerase III, TFIIIC, and the TAF fraction as a source of TFIIIB (29). The second TBP-TAF complex we characterized consists minimally of yTAF170 and TBP (25), yTAF170 is encoded by MOT1, which was originally identified in a genetic screen performed by Thorner and colleagues (34) as a gene encoding a repressor of transcription of numerous genes. Mot1p/yTAF170 has also been independently identified and characterized by Auble and Hahn as ADI, an ATP-dependent inhibitor of TBP binding to DNA (26). This action of Mot1p appears to be polymerase II-specific (35). Finally, we (11) and Reese et al. (12) recently described a multisubunit TFIIID-like complex in yeast. Our work (11) demonstrated that the yTAFs of $M_r = 150, 130, 90, 60, 40, 30$, and 25 kDa, which are present in our TAF fraction, appear to exist in a single TBP-TAF complex with many of the biochemical and genetic hallmarks of
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metazoan TFIIID.

In this report we describe the cloning and characterization of TAF25 a previously uncharacterized yeast gene that encodes a yeast TAF protein that exhibits an apparent M, of 25,000. As was observed for other yeast TAFs (11, 12), we found that the deduced amino acid sequence of yTAF25 exhibits significant similarity to the deduced amino acid sequence of a metazoan TAF, in this case hTAF130 (17). We show here that TAF25 is a single copy essential gene by gene disruption experiments, and through both genetic and biochemical analyses we demonstrate that yTAF25 can interact with itself. Commonmunoprecipitation and transcriptional studies confirm that yTAF25 is in fact a bona fide TBP-associated factor, that it is a subunit of a multiprotein TBP-TAF complex, and that its activity is RNA polymerase II specific. By these criteria the TAF25-encoded protein has been termed yTAF125. Documenting the RNA polymerase II specificity of the yTAFII25-containing TBP and (downstream, GAGATCTAGA(A/G)TA(T/C)TC(G/A/T/C)GC(G/A/T/C)GC; (upstream, GAGAGACTCTAGA(A/G)TA(T/C)TC(G/A/T/C)GC(G/A/T/C)GC; (upstream) dIII site underlined) were generated from yTAF25 genomic DNA. The resulting strain, termed yEK2, Proper integration of the taf25 gene disrupted fragment at the TAF25 locus was verified by genomic Southern blotting and PCR. A yeast expression plasmid encoding yTAF25 was constructed to serve as a covering plasmid for the chromosomal taf25 null allele. This covering plasmid was constructed by doing a BstI/EcoRV genomic fragment containing TAF25 into BstI/EcoRV-digested plasmid pRS416 (Strategene) that had been cut with BamHI and EcoRV. The resulting plasmid, which also carries URA3, was termed pRS416-TAF25. This plasmid was then used to transform yEK2 to uracil prototrophy to produce yeast strain yEK2/pRS416-TAF25. yEK2/pRS416-TAF25 was then sporulated, and the resulting tetrads were dissected. These spores were germinated and subjected to phenotypic testing, and one of the resulting spore clones derived from this dissection was termed yEK16, which has the relevant genotype taf25::TRP1/pRS416-TAF25.

Construction of a Yeast Strain Expressing a Chromosomal Null Mutation of TAF25

Diploid strain SEY6210.5 was used as the parental strain for the disruption of TAF25. PCR oligos were designed to contain 50 nucleotides upstream and 50 nucleotides downstream of the TAF25 sequence both upstream and downstream of the TAF25 ORF. Each PCR oligo also contained 20 nucleotides complementary to TRP1 sequences (upstream, GCGTCGAAAAAAGAAAAGAGG, and downstream, GCAAGTGCACAAACATATTCT) at their 3′ ends. These oligos were in large scale PCR reactions with YEp-pp DNA (43), a plasmid that contains TRP1 sequences. The resulting amplification product thus contained 50 base pairs of TAF25 sequences flanking an intact BglII base pair TRP1 gene. This DNA fragment (taf25::TRP1) was gel-purified and used to generate a TAF25 null allele by transforming strain SEY6210.5 to tryptophan prototrophy; the resulting Trp− strain was called yEK2. Proper integration of the taf25::TRP1 disrupting fragment at the TAF25 locus was verified by genomic Southern blotting and PCR. A yeast expression plasmid encoding yTAF25 was constructed to serve as a covering plasmid for the chromosomal taf25::TRP1 null allele. This covering plasmid was constructed by doing a BstI/EcoRV genomic fragment containing TAF25 into BstI/EcoRV-digested plasmid pRS416 (Strategene) that had been cut with BamHI and EcoRV. The resulting plasmid, which also carries URA3, was termed pRS416-TAF25. This plasmid was then used to transform yEK2 to uracil prototrophy to produce yeast strain yEK2/pRS416-TAF25. yEK2/pRS416-TAF25 was then sporulated, and the resulting tetrads were dissected. These spores were germinated and subjected to phenotypic testing, and one of the resulting spore clones derived from this dissection was termed yEK16, which has the relevant genotype taf25::TRP1/pRS416-TAF25.

Construction of a Yeast Strain Expressing a H4-tagged yTAF25

A plasmid expressing H4-tagged yTAF25 was constructed as follows. The BstI/EcoRV TAF25 gene fragment was cloned into the H3S-containing plasmid pRS413 (Strategene) as above, and an EcoRI site was introduced just 5′ to the TAF25 ATG using site-directed mutagenesis (44) (oligo sequence, TAAATTTAGACTAGATCATGG); a plasmid that contains an N-terminal histidine protein sequence selection (46). The resulting strain, termed yEK2, had the taf25::TRP1 null allele covered by the H3S-marked plasmid pRS413-HA-TAF25.

Bacterial Expression and Purification of yTAF25

The TAF25 ORF was amplified using Pfu DNA polymerase (Stratagene) in PCR reactions (oligos upstream, GAGAGGCTCGAGATTGATCATG; and downstream, CGCTAGCTTAGCTTGTTGAAGGTTCGCTGGG; XbaI site underline) and upstream region (GAATTC/CGCGGCGAT/CTGTCG). The resulting PCR product was digested with HincII and XbaI, gel purified, and then cloned into the expression plasmid pRS413-EcoRI-TAF25 to generate pRS413-HA-TAF25. Plasmid pRS413-HA-TAF25 was introduced into haploid strain yEK16 and exchanged for the EcoRI site of pRS413-EcoRI-TAF25 to generate pRS413-HA-TAF25. The resulting strain was termed yEK20; the taf25::TRP1 null allele was then replaced by the His3-marked plasmid pRS413-HA-TAF25.

The gene encoding yTAF25 was cloned via a PCR strategy based on the amino acid sequence of a yTAF25 tryptic peptide. Two degenerate primers with HindII (upstream) and XbaI (downstream) restriction endonuclease recognition sites (upstream, GAGAAAAGCTTGTGAA/CGGAGGAACTG; downstream, GAGCTAGCTGAGCTAAG; HindII site underline) and (downstream, GAGATCTGGAGATCTGGGCGGATCTGGCGG; XbaI site underline) were generated from yTAF25 peptide 2 (see above). In a first round of PCR the upstream oligo was used together with an oligonucleotide (TAATAGCTACTAGTAC) complementary to the T7 promoter site just downstream of the insertion site of the cDNA into vector pRS316-Gal1/C-DNA (42) using total library DNA as the template. The product of this first PCR reaction was used as the template along with both degenerate oligonucleotides as primers in a second PCR reaction. The products of this PCR reaction were subse-
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TTAGCGTGGCGGA, and downstream, GCCCGGATCCCTTATACACTAA- CGATAAAAGCTGCGGC) or a portion of the Human estrogen receptor (hER) encoding hER amino acids 179–314 (upstream, GAGAGTCT CCGGGAAAAGAGACCTGCTGA, and downstream, GAGAGATCGAAGCTCTTACGAGGGTG (49)). Pfu DNA polymerase (Stratagene) was used in all PCR reactions. PCR products were digested with BamHI and EcoRI and ligated into the hIEL-digested plasmids. Three times triterminalities of all the clones were verified by DNA sequencing. Yeast strain L40 was used as the reporter strain for testing two-hybrid interactions. L40 cells were transformed with various combinations of bait and prey plasmids (see below) using plasmid-borne prototrophic markers to select transformants. At least 10 individual transformants from each strain containing a bait and prey plasmid pair were streaked on a lawn as a patch on a plate with the appropriate medium selection. After growth overnight at 30°C each patch of cells was used to make a pool of yeast transformants. At least 10 individual transformants from each pool were used in all PCR reactions. PCR productswere digested with HindIII and XhoI and ligated into HindIII-XhoI-digested plasmids. The amount of GST or GST-TAF25 protein bound to the resin was estimated via SDS-PAGE/Coomassie Blue staining as described previously (50, 51).

GST Pull-down Experiments

GST-TAF25 and GST resins were prepared from bacterial lysates derived from isopropyl-1-thio-β-β-D-galactopyranoside-induced E. coli harboring plasmids expressing either GST or GST-TAF25 as described elsewhere (48). These resins were stored in column buffer (150 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4·7H2O, 1.4 mM KH2PO4, phosphate-buffered saline, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 mM DTT) containing 0.02% azide at 4°C until used. The amount of GST or GST-TAF25 protein bound to the resin was estimated by boiling a fixed amount of resin in SDS sample buffer and fractionating the sample by SDS-PAGE along with known amounts of a protein standard followed by Coomassie Blue staining.

32P-Labelled HMK-TAF25 probe and varying quantities of unlabeled His6-TAF25 (range, 30–8000 ng) as a competitor. The total amount of protein added was 1 μg/ml bovine serum albumin, 1 mM DTT, 200 μM KAc, 2 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) or a 100-fold molar excess of a peptide recognized by the antibody (sequence, GGGYPYPDPVDPAYGPYPDVDPAGGYGDYPYDPYDAG, HA epitope underlined) in BA200 buffer for 1 hr at room temperature in a total volume of 1.5 ml. Following this preincubation/blocking step the resin was washed six times with 1 ml of BA200 buffer. Next, 250 μl of yEK20 WCE (100 μg of protein/μl) was added to approximately 200 μl of each of these treated resins, and the reactions were placed on a tilttable at 4°C overnight to allow mAb-antigen binding. The supernatants were then harvested by centrifugation, aliquoted, and frozen at −70°C until assayed for TAF25 content and transcriptional activity.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed with yeast WCE as detailed (25) with the following modifications. Antibody and WCE were allowed to incubate overnight on a tilttable rather than for 1 hr at 4°C. Where indicated, blots were subjected to quantitation by exposing the developed blot to a Bio-Rad high intensity imaging screen, which was then analyzed using the Bio-Rad Imager and Molecular Analyst software.

In Vitro Transcription Assays

RNA Polymerase I Assay—WCE (approximately 400 μg of total protein) was added to a reaction mixture mix in a total volume of 24 μl. Specific DNA template (p131–343 (54)) was added and incubated for 5 min at room temperature. tRNAs were then added, bringing the final reaction volume to 30 μl. Final conditions for the transcription reaction were as follows: 10 mM Hepes-KOH, pH 7.6, 10% glycerol, 10 μg/ml bovine serum albumin, 1 mM DTT, 0.1% Nonidet P-40, 20 mM KHPO4, pH 6.8, 7 mM MgCl2, 10 mM MgCl2, 10 mM CaCl2, 100 μM DTT, 5 μg/ml RNasin (Promega), 10 mM MgCl2, 2 mM DTT, 5 μg/ml pyridine-5- and RNA polymerase III-specific (57) transcription assays were carried out as described previously; Gcn4p was purified as described (58) through the ammonium sulfate precipitation step. Specific polymerase II- (56) and RNA polymerase III-specific (57) transcription reactions were performed as described (55), and the amount of appropriate length (50 nucleotides) extended product produced was quantitated by Phosphorimager using the Molecular Analyst software (Bio-Rad), and the yield of extension product was normalized to the yield of the internal control product.

RESULTS AND DISCUSSION

Cloning of the Yeast Gene Encoding yTAF25—As shown in Fig. 1, yTAF25 is the smallest readily visualized TAF within our yeast TAF fraction, which was prepared via immunaffinity chromatography using affinity purified rabbit anti-TBP IgG

Antibodies and Immunological Methods

Polyclonal antibodies directed against yTAF25 were raised in rabbits by Bethyl Labs, Inc. (Montgomery, TX) using purified His6-TAF25 produced in E. coli as antigen. Total immunoglobulins were purified from rabbit serum using ammonium sulfate precipitation and DEAE-cellulose chromatography (53). Anti-yTAF25 antibodies were affinity purified from IgG preparations on a column containing His6-TAF25 coupled to cyanogen bromide-activated Sepharose 4B (Sigma). Anti-influenza virus hemagglutinin (anti-HA) monoclonal antibody, 12CA5, which reacts with the HA-epitope (YPYDVPDYA), was purchased from Boehringer Mannheim.

Immunodepletion Experiments

Monoclonal anti-HA antibody 12CA5 was cross-linked to protein A-Sepharose (2 μg of anti-HA mAb/μl Sepharose) using dimethylpimelimidate (53). This cross-linked immunaffinity matrix was then used to deplete TAF1125 and any other proteins from yeast strain yEK20, which expresses HA-TAF25 as the only form of yTAF25. Prior to incubating the resin with the extract, 600-μl aliquots of the resin were incubated with buffer alone (BA200 buffer: 20 mM Hepes-KOH, pH 7.6, 10% glycerol, 100 μg/ml bovine serum albumin, 1 mM DTT, 200 mM KAc, 2 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) or a 100-fold molar excess of a peptide recognized by the antibody (sequence, GGGYPYPDPVDPAYGPYPDVDPAGGYGDYPYDPYDAG, HA epitope underlined) in BA200 buffer for 1 hr at room temperature in a total volume of 1.5 ml. Following this preincubation/blocking step the resin was washed six times with 1 ml of BA200 buffer. Next, 250 μl of yEK20 WCE (100 μg of protein/μl) was added to approximately 200 μl of each of these treated resins, and the reactions were placed on a tilttable at 4°C overnight to allow mAb-antigen binding. The supernatants were then harvested by centrifugation, aliquoted, and frozen at −70°C until assayed for TAF25 content and transcriptional activity.

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Characterization of yTAF25

yTAF170
yTAF150
yTAF90
yTAF60
yTAF40
yTAF30
yTAF25

FIG. 1. SDS-PAGE analysis of immunopurified yTAFs. A yeast TAF fraction purified using immunoaffinity chromatography with affinity purified rabbit polyclonal anti-TBP IgG was fractionated by SDS-PAGE, and TAF polypeptides were visualized by staining with silver. The arrows and labels indicate TBP-associated factors that consistently coimmunopurify with TBP. Brf1p comigrates with the abundant M.r 25,000 protein. Proteins that do not consistently coimmunopurify with TBP are marked with asterisks.

(see also Refs. 11, 25, 29, and 59). In order to begin the molecular characterization of yTAF25, the TAF fraction was fractionated by SDS-PAGE and electrotransferred to nitrocellulose, and the M.r 25,000 polypeptide was visualized with Ponceau S. The band corresponding to yTAF25 was excised and digested with trypsin, and five of the resulting HPLC-purified tryptic peptides were subjected to amino acid sequencing. Initial data base searches using these derived sequences and the BLAST search algorithms (41) revealed that these sequences were novel.

In order to clone the gene encoding yTAF25, the amino acid sequence of one of the peptides ([K/R]VVLTVNDLSSAVAEY) was used to design degenerate oligonucleotide primer pairs for use in the polymerase chain reaction for amplification of the DNA encoding this peptide. Total yeast cDNA library DNA (42) was used as the template for this PCR reaction. As detailed under "Materials and Methods," two separate rounds of PCR amplification had to be performed prior to obtaining the correct length product, probably due to the degeneracy of the oligonucleotides used as primers. The correct length PCR product was cloned, and the nucleotide sequence of the insert was determined. This nondegenerate DNA sequence was then synthesized, labeled, and used as a probe to screen a yeast genomic library (ATCC 77164). One of the positive clones obtained was selected for sequencing and an ORF of 618 nucleotides was cloned, and the nucleotide sequence of the insert was determined. Recent BLAST searches with this query sequence revealed that it is identical to an uncharacterized ORF found on S. cerevisiae chromosome IV (GenBank accession number S50913).

Characterization of TAF25—When the deduced amino acid sequence of yTAF25 was analyzed, no striking similarities were found between yTAF25 sequences and previously characterized conserved protein structural or functional motifs. However, because the C-terminal region of yTAF25 is somewhat glutamine-rich, there was some similarity observed to other glutamine-rich proteins. While this work was in progress Jacq et al. reported the cloning and characterization of a 30-kDa human TAFII115 termed hTAF25 (17). Direct sequence comparisons of yTAF25 to this similarly sized human TAF protein showed that there are two regions, yTAF25 residues 82–128 and residues 180–198, which show high sequence similarity (59.6 and 52.6% identity, respectively) to portions of hTAFII115 (Fig. 3). However, overall sequence identity between yTAF25 and hTAFII115 is only 27%. The finding of yet another yTAF that is similar at the amino acid sequence level to a metazoan TFIID subunit lends strong additional support to our previously stated hypothesis (11) that the cloned yTAF genes encode subunits of a yeast TFIID multiprotein complex (see also Ref. 12).

TAF25 Is a Single Copy Essential Yeast Gene—As a prelude to gene disruption experiments, we characterized TAF25 genomic DNA and RNA species to rule out the potential for multiple yTAF25-encoding genes. Genomic DNA blots (not shown) indicated that TAF25 is present at a single copy per haploid genome, whereas RNA blots showed that only a single 0.73-kilobase RNA species anneals with TAF25-derived probes.
formed utilized anti-HA antibody and WCE derived from a question. The first immunoprecipitation experiment we per-
cided to use coimmunoprecipitation methods to address this coimmunoprecipitate. We wanted to test if yTAF25 truly was other in a specific complex is to show that the two proteins demonstrating that two proteins are associated with one an-
other are presented the results of the immunoprecipitation experi-
ment designed to determine which of the other yTAFs are in
complex consisting minimally of yTAFs 150, 130, 90, 60, 40, 30, 25, and TBP. To address this question directly using the im-
munoprecipitation methods to address this question. The first immunoprecipitation experiment we per-
formed utilized anti-HA antibody and WCE derived from a yeast strain expressing HA\textsubscript{3}-TBP. Following incubation of the anti-HA antibody with this WCE, the resulting immunoprecipi-
tate was recovered, fractionated by SDS-PAGE, and electro-
transferred to a PVDF membrane. We then determined if yTAF25 coimmunoprecipitated with precipitated TBP by blot-
ing the membrane with anti-TAF25 IgG. WCEs from the same strains used for the left panel were subjected to immunoprecipitation with anti-TAF25 IgG immunoprecipi-
mates were fractionated by SDS-PAGE, electrotransferred to a PVDF membrane, and blotted with anti-HA mAb 12CA5. HA\textsubscript{3}-TBP and HA\textsubscript{3}-TAF25 are indicated by the arrows and labels.

yTAF25 Is Present in a Multiprotein Complex with TFIID-
specific TAFs—We extended our coimmunoprecipitation analyses to determine which if any of the other TAFs in our TAF fraction are directly or indirectly associated with yTAF25. Our previous biochemical studies indicated that yTAF25 is part of a complex consisting minimally of yTAFs 150, 130, 90, 60, 40, 30, 25, and TBP. To address this question directly using the im-
munological reagents developed in the current study, we again used coimmunoprecipitation techniques to ask whether the 25-kDa yTAF polypeptide is also in a complex with other known TAFs. We were aided in these studies by the availability of our collection of different strains of yeasts, which all separately express epitope-tagged versions of either TBP or one of the other known yeast TAFs (i.e. yTAFs 170 (25), 150, 130, 90, 60, 40, 30, 25). We extend our coimmunoprecipitation analyses to determine which if any of the other TAFs in our TAF fraction are directly or indirectly associated with yTAF25.

Not shown. In order to test whether this apparently single copy gene was required for viability, we created the diploid yeast strain yeK2, which contained one wild type and one null mutant copy of TAF25 (i.e., strain yeK2, genotype TAF25/taf25::TRP1). When yeast strain yeK2 was sporu-
lated and the resulting tetrads were dissected, viability segregated 2:2 and all viable spores were Trp\textsuperscript{+}, suggesting that TAF25 is an essential gene. When yeK2 was subsequently transformed to Ura\textsuperscript{+} with the URA3-marked plasmid pRS416-TAF25 and this strain was subjected to sporulation and the resulting tetrads dissected, all four spores were viable, all Trp\textsuperscript{+} spores were Ura\textsuperscript{+}, and the Trp\textsuperscript{+} phenotype segregated 2:2 as expected. These genetic and biochemical analyses show that TAF25, like the previously characterized yeast TAF-encoding genes (TAF170, TAF150, TAF130, TAF90, and TAF60 (11, 25)), is an essential gene indicating an important role for these gene products in yeast cell physiology.

yTAF25 Is a Bona Fide yTAF—One definitive method of demonstrating that two proteins are associated with one an-
other in a specific complex is to show that the two proteins coimmunoprecipitate. We wanted to test if yTAF25 truly was associated with TBP in crude yeast protein fractions and decided to use coimmunoprecipitation methods to address this question. The first immunoprecipitation experiment we per-
formed utilized utilized anti-HA antibody and WCE derived from a yeast strain expressing HA\textsubscript{3}-TBP. Following incubation of the anti-HA antibody with this WCE, the resulting immunoprecipi-
tate was recovered, fractionated by SDS-PAGE, and electro-
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In the experiment depicted in Fig. 5 (top panel), immunoprecipi-
cipitates were formed using WCEs prepared from each of these strains and the anti-HA mAb. An immunoblot of these immu-
noprecipitates shows that the epitope tagged TAFs or TBP are all readily detected using anti-HA mAb. Thus for each strain tested the only reacting species detected in the anti-HA immu-
noblot is the corresponding single, correct sized epitope-tagged polypeptide that the strain expresses. In Fig. 5 (lower panel) are presented the results of the immunoprecipitation experiment designed to determine which of the other yTAFs are in the yTAF25-TBP complex. In this experiment immunoprecipi-
cipitates were again formed using anti-HA mAb, but in this case, the SDS-PAGE fractionated immunoprecipitates were probed with anti-TAF25 IgG. As can be seen in Fig. 5 (lower panel,

\footnote{\textsuperscript{2}K. Gerrish and P. A. Weil, unpublished data.}
Characterization of yTAF\textsubscript{11,25}

**TABLE I**

| Yeast two-hybrid analyses indicate that yTAF\textsubscript{25} interacts with itself |
|---|---|---|
| Strain number | Bait plasmid | Prey plasmid | \(\beta\)-galactosidase (\(\pm\) S.E.) |
| 1 | pLexA-hER(179–341) | pVP16 | 68.55 (11.01) |
| 2 | pLexA-Daughterless | pMyoD-VP16 | 2.79 (1.21) |
| 3 | pLexA-TAF25 | pVP16 | 1.88 (0.17) |
| 4 | pLexA | pTAF25-VP16 | 0.34 (0.09) |
| 5 | pLexA-TAF25 | pTAF25-VP16 | 37.62 (1.58) |

Using this yeast two-hybrid approach, we were not able to detect any interaction between the hER AF-2a region and yTAF\textsubscript{25} (data not shown), which suggests that the structural similarity we noted between yTAF\textsubscript{25} and hTAF\textsubscript{11,30} may not extend to the functional level, although we did not detect any interaction between yTAF\textsubscript{25} and hTAF\textsubscript{11,30} with itself.

**Fig. 6.** GST pull-down experiments show that yTAF\textsubscript{25} interacts directly with itself. Glutathione beads containing approximately 5 \(\mu\)g of either GST-TAF\textsubscript{25} or GST alone were incubated with the indicated amounts of \(^{32}\)P-labeled HMK-His\textsubscript{6}-TAF\textsubscript{25} and washed as described under "Materials and Methods." Resin-bound proteins were separated by SDS-PAGE and visualized by exposing the dried gel to x-ray film. Alternate lanes show the \(^{32}\)P-yTAF\textsubscript{25} protein that bound to GST-TAF\textsubscript{25} (odd-numbered lanes) or to the GST resins (even-numbered lanes). The arrow indicates the labeled yTAF\textsubscript{25} protein bound to the resin. The faster migrating band most likely represents a \(^{32}\)P-yTAF\textsubscript{25} degradation product. The results of the binding assay were quantitated by PhosphorImager and are presented graphically as an inset in the graph. Only the predominant, slower migrating \(^{32}\)P-labeled yTAF\textsubscript{25} species was quantitated.

- **Fig. 5.** yTAF\textsubscript{25} communoprecipitates with TFIIID-specific TAFs. Top panel, HA\textsubscript{3}-TBP or HA\textsubscript{3}-TAF moieties are immunoprecipitated from WCEs prepared from yeast strains expressing the relevant HA\textsubscript{3}-tagged proteins. WCEs were prepared from yeast strains expressing the indicated HA\textsubscript{3}-tagged versions of TBP or TAF. HA\textsubscript{3}-tagged proteins present in the WCEs were immunoprecipitated using anti-HA mAb 12CA5, and the immunoprecipitates were fractionated by SDS-PAGE, blotted to a PVDF membrane, and proteins containing the HA\textsubscript{3} epitope were detected by blotting with anti-HA mAb 12CA5. The arrows and associated labels indicate the various epitope tagged proteins. Bottom panel, yTAF\textsubscript{25} is only detectable in an anti-HA mAb immunoprecipitates derived from strains expressing HA\textsubscript{3}-tagged proteins that are putative TFIIID-specific TAFs. Immunoprecipitates were prepared using anti-HA antibody from the WCEs used in the top panel, and the immunoprecipitates were fractionated via SDS-PAGE and transferred to a membrane, but in this case the blots were probed with polyclonal anti-yTAF\textsubscript{25} IgG. The arrows and associated labels indicate the presence of either native TAF\textsubscript{25} or the more slowly migrating HA\textsubscript{3}-TAF\textsubscript{25}.

- **Fig. 1.** yTAF\textsubscript{25} interacts with itself. Glutathione beads containing approximately 5 \(\mu\)g of either GST-TAF\textsubscript{25} or GST alone were incubated with the indicated amounts of \(^{32}\)P-labeled HMK-His\textsubscript{6}-TAF\textsubscript{25} and washed as described under "Materials and Methods." Resin-bound proteins were separated by SDS-PAGE and visualized by exposing the dried gel to x-ray film. Alternate lanes show the \(^{32}\)P-yTAF\textsubscript{25} protein that bound to GST-TAF\textsubscript{25} (odd-numbered lanes) or to the GST resins (even-numbered lanes). The arrow indicates the labeled yTAF\textsubscript{25} protein bound to the resin. The faster migrating band most likely represents a \(^{32}\)P-yTAF\textsubscript{25} degradation product. The results of the binding assay were quantitated by PhosphorImager and are presented graphically as an inset in the graph. Only the predominant, slower migrating \(^{32}\)P-labeled yTAF\textsubscript{25} species was quantitated.

Determined that hTAF\textsubscript{30} interacts directly with hER amino acids 300–330, which lie within the AF-2 activation domain of the hER (60–62). Interestingly, this precise region (hER amino acids 300–330 termed the hER AF-2a domain) can function as a potent hormone-independent constitutive activator of transcription in yeast (63). We initiated studies to see if the structural similarity noted between yTAF\textsubscript{25} and hTAF\textsubscript{11,30} extended to the functional level. To accomplish this we asked whether we could show that yTAF\textsubscript{25} could bind to hER AF-2a sequences. For this purpose we used the variant of the yeast two-hybrid screening system described by Hollenberg and colleagues (38).

Using this yeast two-hybrid approach, we were not able to detect any indication of an interaction between the hER AF-2a region and yTAF\textsubscript{25} (data not shown), which suggests that the structural similarity we noted between yTAF\textsubscript{25} and hTAF\textsubscript{11,30} may not extend to the functional level, although we did not detect any indication of an interaction between yTAF\textsubscript{25} and hTAF\textsubscript{11,30} with itself.
Immunodepletion demonstrates that a yTAF25-containing complex is required for both basal and activated RNA polymerase II transcription but not for RNA polymerase I or RNA polymerase III transcription. A, immunodepletion was able to remove most of the yTAF25 from a yeast whole cell extract. A yeast WCE expressing HA3TAF25 as the only form of yTAF25 was immunodepleted of yTAF25 and associated proteins using anti-HA mAb 12CA5 cross-linked to protein A-Sepharose as described under “Materials and Methods”. Prior to incubation with WCE, the resin-bound mAb was preincubated with either buffer alone (DEPLETED) or a 100-fold molar excess of specific peptide recognized by the antibody (MOCK DEPLETED). To determine the extent of immunodepletion, the indicated quantities of supernatant resulting after harvesting the immunoprecipitates by centrifugation were fractionated by SDS-PAGE, transferred to PVDF, and immunoblotted using polyclonal anti-TAF25 IgG for detection. The signal on the immunoblot representing yTAF25 is illustrated by the label and the arrow. The relative amount of yTAF25 remaining in the supernatant as compared with untreated WCE was quantitated using a Bio-Rad Imager and is indicated by the numbers below the blot. B, specific RNA polymerase I activity is unaffected by depletion of yTAF25-containing complexes. As described under “Materials and Methods,” the variably treated WCEs were tested in an in vitro RNA polymerase I-specific transcription assay. The results of the assay were scored by primer extension analysis. Following the transcription assay a constant number of cpm of a 300-nucleotide 32P-labeled fragment was included in all the subsequent steps of the analysis as a recovery control. The autoradiograms pictured represent the expected 50-nucleotide extension product for the RNA primary transcript and the 300-nucleotide recovery control. The results were quantitated by imaging and are graphically depicted (bottom). After subtracting for background and normalizing to the recovery control, the PhosphorImager units detected for each extract were as follows: Control, 2335 units; Depleted, 2278 units; and Mock depleted, 2554 units. C, specific transcription by RNA polymerase III is not affected by depletion of yTAF25-containing complexes from WCE. The two WCEs were assayed for specific RNA polymerase III transcription using a tRNALeu3 gene as the template. The autoradiogram shown reveals the products of the transcription assay with primary, partially processed, and mature tRNA Leu3 transcripts being indicated by the arrows and brackets. The signals, representative of transcripts resulting from the UAS Gcn4p-driven template in response to 2, 4, or 6 μl of the treated WCE, are indicated by the arrows. The signals were quantitated by phosphorimaging, and the results are presented graphically (lower panels). After subtracting for background the PhosphorImager units obtained for the basal transcription reactions were as follows: mock depleted extract: 2 μl of WCE, 846 units; 4 μl of WCE, 1464 units; 6 μl of WCE, 2354 units. Depleted extract: 2 μl of WCE, 73 units; 4 μl of WCE, 158 units; 6 μl of WCE, 247 units. D, both basal and Gcn4p-mediated activated RNA polymerase II transcription are severely compromised as a result of depletion of yTAF25-containing complexes. Basal and activated in vitro transcription reactions were performed as described under “Materials and Methods.” The signals, representative of transcripts resulting from the UAS Gcn4p-driven template in response to 2, 4, or 6 μl of the treated WCE, are indicated by the arrows. The signals were quantitated by phosphorimaging, and the results are presented graphically (lower panels). After subtracting for background the PhosphorImager units obtained for the basal transcription reactions were as follows: mock depleted extract: 2 μl of WCE, 846 units; 4 μl of WCE, 1464 units; 6 μl of WCE, 2354 units. Depleted extract: 2 μl of WCE, 73 units; 4 μl of WCE, 158 units; 6 μl of WCE, 247 units.
Characterization of yTAFII25

The yTAFII25 protein is a component of the TFIID complex, which plays a crucial role in transcription initiation. In vitro, yTAFII25 has been shown to directly homomultimerize, forming dimers and higher order oligomers. This self-association is mediated by the yTAF II25 domain and is characterized by high affinity and specificity.

The physiological relevance of yTAF II25-yTAF II5 interactions remains to be determined. It is possible that yTAF II5 could be present in two copies in the yTFIID complex, or alternatively this protein could facilitate interactions between two TFIID molecules. The exact stoichiometry of the various components of the yTAF II5-containing yeast TFIID complex or for that matter the exact stoichiometry of TAF subunits in metazoan TFIIDs remains to be established.

yTAFII25-containing Complexes Are Specific to the Function of RNA Polymerase II—With the exception of the SNAP complex isolated from HeLa cells, which appears to be required for transcription of small nuclear RNA genes by both RNA polymerases II and III (24), all of the other initiation factor TAF-TBP complexes thus far characterized appear to be dedicated to transcription by a single RNA polymerase. With this mind our next goal was to examine the RNA polymerase specificity of yTAF II5-containing TBP-TAF complexes. Before initiating these studies, our prediction was that yTAF II5 would be part of the RNA polymerase II-specific TFIID complex. This hypothesis was based upon two observations. First, there are striking homologies between the sequences of the yeast TAFs (including yTAF II5) of the yTAF II5-TBP core complexes previously described (11, 12) and metazoan TFIID subunits. Secondly we (11) and others (12) have shown that yeast TAF fractions, prepared using two quite distinct affinity chromatographic approaches, could specifically mediate activation by upstream activators in vitro, indicating that the proteins in the yeast TBP-TAF fraction possess TFIID-like coactivator activity. Taken together these findings suggested but did not prove that the yTAF II5-containing TBP-TAF complex represents a yeast TFIID complex and therefore would be RNA polymerase II-specific.

The strategy that we took in order to test this idea was to immunodeplete a yeast WCE of yTAF II5 and associated proteins and then use in vitro transcription assays to measure the ability of the depleted extracts to support specifically initiated transcription by each of the three distinct DNA-dependent RNA polymerases. If the yTAF II5-containing complex is uniquely involved in a TFIID complex, then only RNA polymerase II transcription should be affected by the depletion process. As shown in Fig. 7A treatment of yeast WCE with the anti-HA mAb 12CA5 removed ~70% of the HA-tagged yTAF II5 from the WCE. This depletion was specific because control, “mock depleted” anti-HA mAb (i.e. 12CA5 mAb preincubated with the HA peptide prior to mixing with WCE) failed to remove yTAF II5 from the WCE (compare left and center lanes, Fig. 7A). When this depleted extract was tested for its ability to support transcription by RNA polymerase I (Fig. 7B) or RNA polymerase III (Fig. 7C), only minimal effects were observed, because in both cases the depleted extract was as competent for transcription as was the mock depleted extract. Marked effects, however, were observed when the depleted extract was tested for either basal or Gcn4p-activated RNA polymerase II transcription. Basal transcription mediated by the depleted WCE was reduced to ~10% of the mock depleted control (Fig. 7D, left), whereas Gcn4p-activated transcription was reduced to 15–20% of the control (Fig. 7D, right). This data clearly demonstrated that the yTAF II5-containing TBP-TAF complex was RNA polymerase II-specific, and thus yTAF II5 was designated yTAFII25 to indicate this fact. Furthermore these data argue that the yTAFII25-containing TBP-TAF complex represents the major if not the only form of yTFIID, because if the TAFII25-containing TFIID complex represented only a subset of the total cellular yTFIID, then one would not expect to see such a large decrease in basal RNA polymerase II-specific transcription upon its depletion from the WCE. However, the possibility that other isoforms of TFIID may exist in yeast cannot be eliminated. Apparent multiple forms of TFIID have been reported in the human system (17).

The addition of purified TBP to these depleted and mock depleted WCEs results in the recovery of both the basal transcription signal and surprisingly the signal from Gcn4p activated transcription (not shown). There are several possible explanations for this result. First, because we were only able to deplete ~70% of yTAFII25 and associated polypeptides from the WCE, the addition of TBP could lead to the reformation of a functional TFIID complex, particularly if yTAFII25 polypeptides are in excess relative to TBP. Such reformed TFIID could then
function in both basal and activated transcription. However, recent quantitative immunoblotting experiments in our lab\(^3\) indicate that TBP, TAF\(_25\), and TAF\(_{112}\) are roughly stoichiometric in yeast cells, a result consistent with functional studies of TBP content in vivo performed by others (68–70). Second, the transactivation event observed with the depleted WCE upon TBP addition could theoretically be fundamentally different in mechanism from that observed prior to depletion or in the mock depleted WCE. Two groups recently identified and characterized “holoenzyme” forms of yeast RNA polymerase II, which are essential for mediating transactivation in vitro (71, 72) and in vivo (73). Holoenzyme preparations can support Gcn4p (the activator used in our studies)-mediated transactivation in reconstituted transcription systems dependent on TBP (72). Because neither TBP nor TAFs appear to be components of the multisubunit RNA polymerase II holoenzyme (11, 59, 71, 72) and because the amount of this form of enzyme, which is competent for transcriptional activation, would be unchanged in our depleted WCE, transactivation events could still occur in our “depleted” WCEs if TBP is re-supplied. Finally, extensive genetic and biochemical analyses have implicated various coactivator or adaptor molecules in transcriptional regulatory proteins and with TBP, thereby potentially obviating any requirements for TAFs for transducing signals between DNA bound transcription factors and the activation domains of transcriptional regulatory proteins. Molecules that have been well characterized are Ada2p and Ada3p (74, 75). We thank Steve Buratowski, Todd Graham, and the University of California at Berkeley, for his help in obtaining the amino acid sequence of yTAFII25. We thank Steve Buratowski, Todd Graham, and members of our lab for freely sharing reagents, strains, and, most of all, constructive criticism and suggestions during the course of this work.

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\(^3\) Y. Bai and P. A. Weil, unpublished data.
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