Direct TFIIA-TFIID Protein Contacts Drive Budding Yeast Ribosomal Protein Gene Transcription*

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Received for publication, May 21, 2013, and in revised form, June 28, 2013 Published, JBC Papers in Press, June 28, 2013, DOI 10.1074/jbc.M113.486829

Background: Enhancer-bound transactivators communicate with promoter-bound transcription machinery through specific interactions.

Results: We identified and dissected the interacting surfaces of TFIIA, TFIID, and the transactivator Rap1.

Conclusion: Rap1 and TFIIA make specific contacts with the Taf4 subunit of TFIID, leading to Rap1-controlled transcription of ribosomal protein coding genes.

Significance: Knowledge of the mechanisms of mRNA gene transcription activation is crucial for understanding gene control.

We have previously shown that yeast TFIID provides coactivator function on the promoters of ribosomal protein-encoding genes (RPGs) by making direct contact with the transactivator repressor activator protein 1 (Rap1). Further, our structural studies of assemblies generated with purified Rap1, TFIID, and TFIIA on RPG enhancer-promoter DNA indicate that Rap1-TFIID interaction induces dramatic conformational rearrangements of enhancer-promoter DNA and TFIID-bound TFIIA. These data indicate a previously unknown yet critical role for yeast TFIIA in the integration of activator-TFIID contacts with promoter conformation and downstream preinitiation complex formation and/or function. Here we describe the use of systematic mutagenesis to define how specific TFIIA contacts contribute to these processes. We have verified that TFIIA is required for RPG transcription in vivo and in vitro, consistent with the existence of a critical Rap1-TFIIA-TFIID interaction network. We also identified essential points of contact for TFIIA and Rap1 within the Rap1 binding domain of the Taf4 subunit of TFIID. These data suggest a mechanism for how interactions between TFIIA, TFIID, and Rap1 contribute to the high rate of transcription initiation seen on RPGs in vivo.

The eukaryotic general transcription factors TFIIA, -B, -D, -E, -F, and -H contribute importantly to the process of accurate initiation of mRNA synthesis by RNA polymerase II (1, 2). However, depending on in vitro transcription assay conditions, there is not always a strict requirement for all six general transcription factors (3), and consistent with this observation, genome-wide approaches have documented variable contributions of general transcription factors, particularly TFIID, to gene-specific transcriptional programs (4–8). Interestingly, the canonical TFIID complex can be altered, diminished, or abolished during the process of cellular differentiation (9–11). Collectively, these observations point to a context-specific role for TFIID and are also consistent with models suggesting a coregulatory role for TFIID, where this multisubunit complex directly engages activator proteins to drive dedicated transcriptional programs (12–14). Consequently, the exact relationships between gene promoter architecture, chromatin modifications and conformation, activator-TFIID interactions, dependence on TFIID function, and mechanisms of TFIID-containing PIC formation and function remain elusive.

The evolutionarily conserved TFIID complex is composed of the TATA-box DNA-binding protein (TBP) and 14 TBP-associated factors (Tafs) (15). Tafs provide promoter-DNA binding (16–20), chromatin-modifying (21), and/or modified histone-binding activities (22, 23), as well as protein-protein interaction domains that directly bind activators (24, 25). TFIIA (26–29), Mediator (30), and other general transcription factors. The involvement of TFIID in these extensive protein-DNA and protein-protein interactions indicates that TFIID plays crucial roles in integrating numerous regulatory inputs to modulate transcription. TFIIA-promoter DNA binding is inhibited by the binding of the Taf1 N-terminal domain (TAND) to the DNA binding domain of TBP (31, 32). TAND inhibition is alleviated by TFIIA-TBP interaction, a result explaining the TFIID dependence of DNA binding by TFIIA (15, 33, 34). Direct activator-Taf interactions have been documented for numerous higher eukaryotic activators (14) but were only recently documented for yeast activators and yeast TFIID (24, 35–37).

Consistent with the overall similarities of TFIID subunit composition, sequence conservation, and function, electron microscopy (EM) of yeast and human complexes reveal a simi-
lar trilobed architecture (38–40). Biochemistry, genetics (15), and EM difference mapping with subunit-specific antibodies have been used to determine the stoichiometry and location of Tafs within budding yeast TFIID. These data have greatly facilitated the structural characterization of promoter-, activator-, and TFIIA-TFIID interactions and conformational rearrangements of complexes containing these molecules (40–43). Recent high resolution mapping of a 7-Taf recombinant human TFIID subcomplex indicates similar patterns of Taf location with the TFIID holocomplex (44). Collectively, these structural studies allow for the connection of biochemical data with molecular genetic characterization of conserved Taf/TFIID protein-protein interactions (e.g. the binding of activators with TFIID and TFIIA). Such experiments can be accomplished with ease and high precision in the yeast system due to the many powerful molecular genetic tools available in *Saccharomyces cerevisiae*.

RPGs are the most vigorously transcribed genes in exponentially dividing yeast cells, and their transcription is strictly TFIID-dependent (4, 5, 8, 45–48). We have previously shown that Rap1 directly interacts with TFIID subunits Taf4, Taf5, and Taf12, through sequences we termed Rap1 binding domains (RBDs). These data show that direct, high affinity, specific interactions between Rap1 and the Taf4 RBD and the Taf5 RBD are critical for RPG transcription (24, 35).

Further, we have recently expanded the use of RPGs to investigate TFIID function through a series of EM studies that defined the structure of several minimal pre-PIc assemblies containing TFIID, TFIIA, Rap1, and RPG enhancer-promoter DNA (42). Intriguingly, we found that the intervening DNA between Rap1 bound to the UAS<sub>Rapi</sub> enhancer site and TFIID bound to the promoter forms an extended loop and, concomitantly, a conformational change in TFIID within the quarter-nary Rap1-TFIID-TFIID-DNA complex. The resulting DNA “locked” features of this complex probably contribute critically to the high levels of RPG transcription seen *in vivo*. Here we report the extension of these analyses, where we examine the genetic and biochemical relationships between Rap1, TFIIA, and TFIID. In this study, we have systematically probed the TFIIA and Taf4 amino acid residues required for RPG transcription in order to identify the surface(s) that participate in protein-protein interactions between TFIIA, Rap1, and TFIID while simultaneously generating key reagents for further dissection of these regulatory protein-protein and protein-DNA interactions.

We have found that TFIIA makes critical contributions to RPG transcription through a variety of contact points in the N-terminal domain of the Toa2 subunit. We identified direct TFIIA-TFIID interactions through the RBD of Taf4, the domain that we had previously characterized as a conserved, high affinity Rap1 binding site (24). Finally, we defined *taf4* RBD mutants that compromise growth, TFIID-TFIID binding, Rap1-TFIID binding, and RPG transcription *in vivo* and *in vitro*. These phenotypes can be partially alleviated by overexpression of TFIIA. Collectively, our data indicate that Taf4 is vital for TFIID-Rap1 and TFIID-TFIIA interactions. Hence, this TFIID-specific subunit represents a critical functional nexus for RPG transcription regulation and, consequently, cellular proliferation.

**EXPERIMENTAL PROCEDURES**

**TOA1, TOA2, and TAF4 Yeast Strains and Expression Vectors**—The TOA1 shuffling strain (genotype MATa <em>leu2Δ0 ura3Δ0 his3Δ1 lys2Δ met15Δ toa1Δ::HPMX4 p416ADH TOA1) was derived from BY4741 (Open Biosystems). The TOA2 shuffling strain (genotype MATa <em>leu2Δ0 ural3Δ0 his3Δ1 lys2Δ MET15 toa2Δ::HPMX4 p416ADH TOA2) was derived from BY4742. Vector constructs targeting TOA1 or TOA2 for deletion were prepared by PCR amplification from genomic DNA derived from strain S288C using primers containing SpeI/EcoRV (5’) or BglII/HindIII (3’) sites that were subcloned into pAG32 (49). PCR-derived fragments included 0.5–1 kilobase of sequence up- and downstream of TOA1 or TOA2 coding sequence. Covering plasmids in p416 ADH (50) included complete TOA1 or TOA2 open reading frames with natural start and stop codons, inserted as PCR-derived BamHI/Xhol fragments. To create strains for plasmid shuffle assays, SpeI/HindIII-digested targeting constructs were mixed with the corresponding p416 ADH TOA covering plasmid, transformed into BY4741 and BY4742, and plated on synthetic complete medium plates lacking uracil (SC–Ura). Colonies were replica-plated onto yeast extract peptone dextrose plates containing hygromycin B (49). Resistant colonies were stricken onto hygromycin plates and then tested for 5-fluoroorotic acid (5-FOA) sensitivity on SC + 5-FOA plates (51). Multiple 5-FOA-sensitive clones were tested by a plasmid shuffle assay for complementation at 37 °C by p413HA<sub>3C</sub> (protease 3C) TOA1 or p415 HA<sub>3NLS</sub> TOA2 in parallel to the corresponding empty vector p413HA<sub>3C</sub>-TOA1 or p415 HA<sub>3NLS</sub> TOA2. Expression vectors were created by inserting genomic 5’-flanking sequence as polymerase chain reaction (PCR)-derived ScaI/Spel fragments into pRS413 or pRS415 (52). The corresponding 3’-flanking DNA fragments were inserted as PCR-derived Xhol/KpnI fragments. Yeast codon-optimized single HA, triple HA-NLS (nuclear localization sequence), or triple FLAG epitope tag sequences were inserted as SpeI/BamHI oligonucleotide duplexes in order to facilitate N-terminal protein tagging for detection and immunoprecipitation. BamHI/Xhol fragments encoding TOA1 or TOA2 originated from the corresponding p416ADH construct. Insertion resulted in the final covering plasmid p413 HA<sub>3C</sub>-TOA1 or p415 HA<sub>3NLS</sub> TOA2.

PCR-mediated site-directed mutagenesis was performed using the gene splicing by overlap extension (SOEing) method (53) with recombinant *Pfu* DNA polymerase and TOA1 or TOA2 templates subcloned as BamHI/Xhol fragments into the pBG101 bacterial T7 expression vector (see the Vanderbilt University Center for Structural Biology Web site), including sequence for an N-terminal His<sub>_5</sub>-GST tag. An oligonucleotide corresponding to the GST coding sequence was used as a universal 5’ mutagenesis primer, and the T7 terminator reverse primer was used as a universal 3’ primer. Final mutated PCR products were reintroduced as BamHI/Xhol fragments into p413HA<sub>3C</sub> TOA1 or p415 HA<sub>3NLS</sub> TOA2 that lacked the TOA1 or TOA2 coding sequence.
The TAF4 shuffling strain (genotype MATa leu2Δ0 ura3Δ0 his3Δ1LYS2 met15Δ0 taf4Δ::KANMX6 p416ADH-TAF4) was derived from BY4741 (24). Targeted deletion constructs and covering plasmids in pRS413 and pRS415 were created essentially as described for TOA1 and TOA2, except the TAF4 ORF was inserted as a BamHI/SalI fragment. Site-directed mutagenesis of TAF4 was carried out as above. High copy plasmids containing TOA1 (BamHI/Xhol), TOA2 (BamHI/Xhol), SPT15 (TBP, BamHI/Xhol), TAF4 (BamHI/SalI), TAF11 (NdeI/Klenow/Xhol), or RAP1 (NdeI/Klenow/Xhol) were based on p423 ADH (50). The high copy plasmid expressing both TOA1 and TOA2 was created by insertion of a Sall/PstI TOA1 genomic fragment and a Pst TOA2 genomic fragment into pRS425 (54, 55), pRS316 TOA1 and TOA2 genomic clones were recovered from yeast strains SHY93 and SHY94 (56), respectively, kind gifts of Dr. Steve Hahn. pRS425 TOA variants were isolated that contained either TOA1, TOA2, or both TOA1 and TOA2, with the latter inserted in both possible orientations; only those clones containing TOA1 and TOA2 inserted in the 3′-3′ end-to-end orientation were functional for suppression of taf4 mutants. All of the expression constructs used in this study were sequence-verified by DNA sequencing and aligned to the S288C reference genome. Additional details of oligonucleotide and vector sequences are available on request.

Plasmid Shuffle Assays—Shuffling strains were transformed with the appropriate covering plasmids encoding WT or variant proteins. Transformants were plated on SC selective media, either SC−His (TOA1 and TAF4 genetic/growth assays), SC−Leu (TOA2 assays), or SC−His−Leu (toa2- and taf4-TFIIA high copy suppression genetic/growth assays). Single colonies were picked to a liquid culture of the appropriate selective medium and incubated at room temperature for 7–10 days to reach stationary phase. A portion of each culture was transferred to one well of a 96-well Bacti-plate (NUNC) and diluted serially 1:4. A 96-well pinning tool was used to transfer cells from each well to plates containing appropriate selective medium supplemented with 5-FOA. Replicates were plated and incubated at room temperature (20–23°C) or 30, 34, or 37°C. Plates were photographed after 3 days of incubation except for the room temperature plate, which was photographed after 7 days. Single colonies from the room temperature plate were inoculated into yeast extract peptone dextrose liquid cultures and used as a source of preinoculum for temperature shift, immunoblots, and immunoprecipitations.

Antibodies—Anti-Taf and anti-Rap1 rabbit polyclonal antibodies were described previously and were used as total IgG prepared by ammonium sulfate precipitation of antisera and subsequent Protein A affinity chromatography (35, 57). Secondary detection was with goat anti-rabbit Fc-horseradish peroxidase (HRP) conjugate (Pierce). Anti-HA used for immunoprecipitation was Protein A-purified and originated from the 12CA5 hybridoma (Vanderbilt Antibody/Protein Resource). Anti-FLAG for immunoblotting was the M2-HRP conjugate (Sigma), whereas standard M2 was used for immunoprecipitation. Polyclonal anti-TFIIB antisera were generated (Bethyl Laboratories) by immunization of rabbits with purified TFIIB complex renatured from recombinant denatured subunits. IgG was prepared as above. Immunoprecipitation and immunoblotting were performed as described previously (57).

Temperature Shift, RNA Isolation, Primer Extension, and RNA Sequence (RNA-Seq) Analyses—Paired yeast extract peptone dextrose cultures of each strain were grown at room temperature to mid-log phase, and one was subjected to temperature shift at 37°C (24, 35, 58). After 2 h of additional incubation, cultures were harvested by filtration, and RNA was isolated immediately by extraction of cells on the filters with hot acidic phenol, essentially as described (59, 60). Transcript abundance measurement by multiplex 5′ primer extension was performed as described previously (24). RNA samples used for deep sequencing were treated with DNase I to remove residual genomic DNA. The quality and quantity of each RNA preparation were determined by measuring A260/A280 on a Nanodrop ND-1000 spectrophotometer and then validated with the Qubit fluorometer RNA assay. RNA integrity was determined by capillary electrophoresis using an RNA Nano chip on an Agilent BioAnalyzer. Sequencing libraries were generated using the Illumina TruSeq RNA Sample Prep kit with modifications to the manufacturer’s protocol. Briefly, poly(A)+ mRNA was isolated from 500 ng of total RNA on oligo(dT) magnetic beads. Purified RNA was fragmented, annealed with random hexamer primers, and applied as template for first strand cDNA synthesis by SuperScript II reverse transcriptase (Invitrogen). Residual dNTPs were removed using the RNA Clean and Concentrator-5 kit (Zymo Research) and eluted in 20 μL of water. Second strand cDNA was synthesized using the NEBNext mRNA second strand synthesis module (New England Biolabs) using the dNTP-free reaction buffer and a dTTP-free dNTP mix containing dUTP (U.S. Biochemical). Double-stranded cDNA was purified with AMPure XP beads and applied to the standard Illumina library protocol including end repair, dA tailing, and ligation with Illumina sequencing adapters. Prior to PCR, dsDNA libraries were treated with USER enzyme to introduce single strand breaks (New England Biolabs). Following PCR enrichment, libraries underwent fragment size confirmation using an HS DNA chip on an Agilent BioAnalyzer and were quantified using the Illumina library quantification kit (KAPA). Library pools underwent cluster generation on an Illumina cBot HiSeq 2000 instrument at the Vanderbilt Genome Technologies for Advanced Genomics Core Facility (VANTAGE, Nashville, TN). Raw sequencing reads were processed through CASAVA-1.8.2 for FASTQ conversion and demultiplexing. The CASAVA quality filter was used, and pass filter reads were provided. Transcript-specific reads/kilobase/million reads (RPKM) values were defined using the QSeq routine of ArrayStar3 software.

Recombinant Protein Expression and Purification—A TOA1 fragment was digested with BamHI, blunted with the Klenow fragment of Escherichia coli DNA polymerase I, and then cut with XhoI and inserted into pBG100 cut with NdeI, blunted with Klenow, and cut with XhoI. The resultant vector includes the T7 promoter and ribosome binding site element immediately upstream of TOA1 without any additional encoded N- or C-terminal amino acids. TOA2 bacterial expression vectors included BamHI/XhoI fragments from TOA2 or toa2 yeast vec-
tors inserted into the equivalent sites of pBG100, resulting in encoded Toa2 with an N-terminal His$_6$ tag. Expression of Toa1, Toa2, and Toa2 variants was performed in parallel with room temperature cultures using the Rosetta E. coli DE3 strain (EMD Biosciences) and an autoinduction medium system (24, 61). Induced cell pellets from 1-liter cultures were harvested by centrifugation and immediately resuspended in buffer containing 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 10% glycerol, and 250 µg/ml egg white lysozyme. After 30 min at room temperature, cell suspensions were rapidly frozen at −80 °C. Material was thawed, and Triton X-100 was added to 1% final concentration. Lysate viscosity was reduced by sonication with a model 250 Branson sonicator equipped with the large tip. Insoluble material was pelleted by centrifugation, supernatant was discarded, and the pellet was resuspended in buffer containing 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol. Insoluble material was again pelleted, and this wash step was repeated twice more. Two more wash steps were performed with buffer containing 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, and 10% glycerol, followed by two final wash steps with 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 5 M urea, and 10% glycerol. Inclusion body proteins were solubilized in 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 7 M urea, 1 M DTT, and 10% glycerol. Denatured Toa1 was mixed with Toa2 or Toa2 variants at a 2:1 Toa1/Toa2 molar ratio and diluted with solubilization buffer to 45 ml at 0.25 mg/ml final protein concentrations and then dialyzed over 24 h against 4 liters of 25 mM HEPES, pH 7.6, 450 mM NaCl, 50 mM NH$_4$Cl, 20 mM imidazole, and 10% glycerol. Renaturation of all TFIIA forms was performed in parallel. Dialyzed material was centrifuged to remove precipitated protein, and cleared supernatant was applied to nickel-nitriiotriacetic acid columns equilibrated and washed with 25 mM HEPES, pH 7.6, 100 mM sodium acetate, 20 mM imidazole, 10% glycerol and eluted with the same buffer containing 200 mM imidazole. TFIIA variants were concentrated using centrifugal filtration devices (Amicon; 10,000 molecular weight cut-off) and subjected to size exclusion chromatography using centrifugal filtration devices (Amicon; 10,000 molecular weight cut-off) and subjected to size exclusion chromatography with a model 250 Branson sonicator equipped with the large tip. Insoluble material was pelleted by centrifugation, supernatant was discarded, and the pellet was resuspended in buffer containing 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol. Insoluble material was again pelleted, and this wash step was repeated twice more. Two more wash steps were performed with buffer containing 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, and 10% glycerol, followed by two final wash steps with 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 5 M urea, and 10% glycerol. Inclusion body proteins were solubilized in 30 mM Tris, pH 8.0, 450 mM NaCl, 50 mM NH$_4$Cl, 1 mM EDTA, 7 M urea, 1 M DTT, and 10% glycerol. Denatured Toa1 was mixed with Toa2 or Toa2 variants at a 2:1 Toa1/Toa2 molar ratio and diluted with solubilization buffer to 45 ml at 0.25 mg/ml final protein concentrations and then dialyzed over 24 h against 4 liters of 25 mM HEPES, pH 7.6, 450 mM NaCl, 50 mM NH$_4$Cl, 20 mM imidazole, and 10% glycerol. Renaturation of all TFIIA forms was performed in parallel. Dialyzed material was centrifuged to remove precipitated protein, and cleared supernatant was applied to nickel-nitriiotriacetic acid columns equilibrated and washed with 25 mM HEPES, pH 7.6, 100 mM sodium acetate, 20 mM imidazole, 10% glycerol and eluted with the same buffer containing 200 mM imidazole. TFIIA variants were concentrated using centrifugal filtration devices (Amicon; 10,000 molecular weight cut-off) and subjected to size exclusion chromatography on a calibrated 3 × 30-cm Superose 12 column in 25 mM HEPES, pH 7.6, 300 mM sodium acetate, and 10% glycerol. Peak Toa1/Toa2-containing fractions corresponding to the expected molecular mass of TFIIA (~48 kDa) were combined and stored at −80 °C.

BamHI/Sall fragments from p413 HA$_5$NLS TAF4 or taf4 were transferred to pLM302, resulting in T7 expression vectors expressing Taf4 with N-terminal His$_6$-maltose-binding protein (MBP) tags. pLM302 TAF4 vectors were mixed with pACYC184 11b TAF12 (62) and transformed into E. coli BL21 DE3 cells containing the pSC101 RIL vector (additional arginine, isoleucine, leucine tRNA genes, compatible pSC101 origin, streptomycin resistance gene).3 Coexpression of His$_6$-MBP-Taf4 with Taf12 occurred in 250-ml cultures grown in autoinduction medium at room temperature. Cells were harvested and resuspended in buffer containing 25 mM HEPES, pH 7.6, 250 sodium acetate, 20 mM imidazole, 0.1% Triton X-100, and 10% glycerol along with a panel of protease inhibitors. Resuspended cells were lysed by sonication, and insoluble material was removed by centrifugation. The soluble supernatant was subjected to nickel-nitriiotriacetic acid chromatography, washed with the lysis buffer, and eluted with the lysis buffer containing 200 mM imidazole. Eluted material was applied to SP-Sepharose columns (GE Healthcare); washed with 25 mM HEPES, pH 7.6, 250 mM sodium acetate, 0.1% Triton X-100, 10% glycerol; and eluted with the same buffer containing 1 M sodium acetate. Peak MBP-Taf4/Taf12-containing fractions were combined and stored at −80 °C. His$_6$-Rap1 from pET28A RAPI or His$_6$-GST-Rap1 expressed from pBG28A RAPI was purified essentially as described above for Taf4/ Taf12 heterodimers. TBP was prepared according to established procedures (63, 64).

**Electrophoretic Mobility Shift DNA Binding Assays (EMSA)**—The ability of purified recombinant WT and Toa2 variant TFIIA to stabilize a TBP-TATA DNA complex was tested by EMSA using a 32P-labeled (specific activity ~3000 cpm/fmol) Ad2 major late promoter TATA DNA probe (WT, CGAAGGGGGCTATAAAAGGGG; TATA box in boldface type; mutant TATA-box, CGAAGGGGGCTAAGAAAGGG; mutations underlined). 25-µl reactions contained 20 mM HEPES, pH 7.9, 50 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 0.01% Nonidet P-40 (Pierce SurfactAMPS), 10% glycerol, 1 mM DTT, 2.5 µg of BSA, 125 ng of poly(dG-dC), and 25 fmol of [32P]TATA DNA. 15 fmol of purified yeast TBP and/or 2.5, 15, or 30 fmol of WT or mutant TFIIA were added, and reactions were incubated for 30 min at 20 °C (room temperature). Where applicable, unlabeled competitor DNAs were added at a 100-fold excess relative to the 32P-labeled probe DNA. Following binding, reactions were fractionated on magnesium-free, native 6% polyacrylamide gels (prepared from a 30:8% acrylamide/bisacrylamide stock) run in 1× TBE (90 mM Tris, 90 mM borate, 2 mM EDTA), dried, and exposed to K-imaging screens for quantification.

**In Vitro Transcription Assays**—Whole cell extract (WCE; final protein concentration 35 mg/ml) in 20 mM HEPES, pH 7.9, 50 mM potassium acetate, 10 mM MgSO$_4$, 10 mM EGTA, 20% glycerol, and 5 mM DTT) was prepared, antibody-depleted of Rap1 and TFIIA, and assayed by G-free template transcription as described previously (15, 35) with a few modifications to optimize signals; 30-µl assays involved a 10-min, 20°C preincubation of WCE (525 µg) and DNAs in the absence of NTPs to allow PIC formation and 30 min of transcription at 20°C in the presence of 400 µM ATP, 400 µM CTP, 2 µM cold UTP, 100 µM 3′-O-methyl-GTP, and 5 µCi of [α-32P]UTP (~800 Ci/mmol). Final solution conditions were 30 mM HEPES, pH 7.9, 75 mM potassium acetate, 12 mM magnesium acetate, 0.75% polyethylene glycol, 13.3% glycerol, 2.5 mM DTT, 5 µg/µl creatine kinase, 25 µM phosphocreatine, 20 units of RNAsin (Promega), 150 units of ribonuclease T1, 500 ng of UAS$_{GAL}$, and 50 ng of purified recombinant Gal4-VP16. The content of Rap1 and TFIIA in WCE, as determined by quantitative immunoblotting using purified recombinant Rap1 and TFIIA as standards was 45 and 1.5 ng/µl, respectively. Where add-back experiments were per-

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3 J. H. Layer, unpublished observations.
formed, the amounts of purified recombinant WT and mutant TFIIA and WT Rap1 added to transcription assays were 1× for Rap1 and 10× for TFIIA, relative to the amount of these proteins present in the original control WCE; these amounts were empirically determined to be the minimal amount of protein that generated the maximal signals. The Rap1-driven transcription template contained two copies of the Rap1 enhancer upstream of the PGK1 core promoter (35, 42, 65) fused to the 378 bp G-free cassette derived from pMLΔ53 (66), whereas the Gal4-driven template contained two copies of UAS<sub>GAL</sub> upstream of the CYC1 promoter fused to the 278-bp G-free cassette of pJΔ70 (67). Transcription was stopped by the addition of 270 μl of 150 mM sodium acetate, pH 5.5, 0.1% SDS, and 25 μg of <i>E. coli</i> tRNA. RNA was purified by sequential phenol/CHCl<sub>3</sub> and CHCl<sub>3</sub> extraction and ethanol precipitation. RNA samples were vacuum-dried and resuspended in 20 μl of 90% (v/v) deionized formamide, 50 mM EDTA, pH 7.9, 0.1% (v/v) SDS, 0.05% xylene cyanol, and 0.05% bromphenol blue. Dissolved samples were heated for 2 min at 100°C and held at this temperature until loaded on denaturing 6% polyacrylamide gels (prepared from a 30:0.8% acrylamide/bisacrylamide stock) containing 7 μl urea and buffered with 1× TBE; gels were run at a constant power sufficient to maintain gel temperatures of 50–55°C. Gels were soaked in H<sub>2</sub>O for 10 min on a gyro shaker to remove urea and vacuum-dried, and <sup>32</sup>P-labeled RNAs were visualized by exposure to an Eastman Kodak Co. K-screen and imaged with a Bio-Rad PhosphoFX imager. Data were quantified using QuantityOne software.

**In Vitro Pull-down Assays—**GST pull-down assays were conducted as 100-μl reactions containing 20 μl HEPES, pH 7.6, 150 mM sodium acetate, 0.05% Triton X-100, 10% glycerol, 1 mM DTT, 25 ng/μl BSA, 5 μl of glutathione-Sepharose Fast Flow (GE Healthcare), 7.5 pmol of GST or 2.5 pmol of GST-Rap1, and between 2.1 and 42 pmol of His<sub>6</sub>-MBP-Taf4/Taf12 variants as input. Binding proceeded for 1 h at room temperature, followed by three washes with GST pull-down buffer. Bead-bound material was eluted with 1× NuPAGE sample buffer, resolved on 4–12% NuPAGE gels, stained with Sypro Ruby (Invitrogen), and imaged with a Bio-Rad PhosphoFX imager, and binding data were quantified with QuantityOne Software. MBP pull-down reactions were similar and used 5 μl of amylose-agarose (New England Biolabs), 8 pmol of MBP or 4 pmol of MBP-Taf4/Taf12, and input of between 4 and 70 pmol of TFIIA. Binding data were analyzed and plotted using GraphPad Prism.

**RESULTS**

**Identification and Characterization of Novel toa1 and toa2 Mutants—**In our previous cryo-EM studies of Rap1-TFIIA-TFID-RPG DNA complexes, we generated two particular yeast TFIIA variants that contained alanine (Ala) substitutions in the Toa2 subunit (42). Yeast Toa2 Y10A and yeast Toa2 F71A were chosen based on equivalent Y6A and F67A substitutions in human TFIIAγ that cause defects in activator-dependent but not basal transcription in vitro, without apparent effects on either TFIIA stability or the formation of TFIIA-TBP-DNA ternary complexes (68). These published results suggest that Y6A and F67A substitutions in human TFIIAγ cause defects in protein-protein interactions that probably involve activators and/or coactivators, such as TFID (68). When we compared Toa1-Toa2 heterodimeric yeast TFIIA preparations carrying the human equivalent Toa2 Ala substitutions (i.e. yeast Y10A and/or F71A) with WT TFIIA, we observed reduced Rap1-, TFIIA-, and TFIIID-dependent looping of RPG DNA (42), results indicating that TFIIA interacts directly with TFID possibly with Rap1 to drive RPG transcription. We thus reasoned that identifying additional mutant variants of TFIIA (i.e. mutations within Toa1 and Toa2 subunits) and mutations within a/the (putative) TFIIA TFID-Taf subunit interaction partner, we would both gain greater insight into transcriptional trans-activation mechanisms and generate valuable tools for the molecular dissection of the coactivator functions of TFIIA and TFID.

Before embarking on a systematic molecular genetic dissection of TFIIA-encoding subunits, however, we needed to demonstrate that the biochemical/structural defects we had observed with the Y10A and F71A Toa2/TFIIA variants in our EM studies correlated with in vivo growth deficiencies, as would be predicted if these interactions contributed to RPG transcription, which is tightly coupled to growth rates. Because TOA2 (and TOA1) is a single copy essential yeast gene (69), we constructed a pseudodiploid TOA2 wild type (WT) strain for plasmid shuffle assays in order to test for any growth defects associated with either (or both) of our Toa2 alanine substitutions. We observed that, indeed, both Y10A and F71A Toa2 variants cause temperature-sensitive growth (Ts<sup>+</sup>), whereas the Y10A/F71A double mutant was inviable; all proteins were as stable as WT Toa2 (Fig. 1A) and assemble to form TFIIA. Hence, steady state protein levels do not explain the observed growth deficiencies of these strains. Rather, collectively, these data indicate that Toa2 amino acid residues Tyr<sup>19</sup> and Phe<sup>21</sup> do indeed contribute importantly to TFIIA function in vivo and motivated us to systematically analyze both TOA1 and TOA2 by alanine-scanning mutagenesis. A number of Ts<sup>+</sup> toa1 and toa2 mutants have been described previously; these include toa1-25 (K255A/R257A/K259A) and toa2-3 (D21A/D24A) (56), toa2127A or I27K (26), and toa2L23A, D24A, D24L, I27A, E33L, or A34L (27). However, these mutants were not identified through a comprehensive alanine-scanning mutagenesis approach.

The TFIIA-TBP-TATA co-crystal structure (Protein Data Bank structure 1RM1) reveals a two-domain TFIIA Toa1-Toa2 heterodimeric assembly. One domain is composed of interacting hydrophobic C-terminal Toa1 and Toa2 β-sheets that form an extensive TBP interaction surface as well as a small region that binds TATA DNA (70–72). A second domain is generated by interactions between two N-terminal Toa1 α-helices with two Toa2 N-terminal α-helices to generate a four-helix bundle. It has been proposed that this four-helix bundle is an extended scaffold that makes important interactions with coregulatory factors, including NC2 (negative cofactor 2), Tafs, and SAGA (26, 27, 70, 72–74). To elucidate which TFIIA amino acids contribute to transcription, particularly to RPG transcription, we created non-overlapping three-codon Ala block mutations that span the length of the TOA1 gene as well as similar two-codon Ala substitutions spanning TOA2.
A number of the \textit{toa1} Ala scan mutants caused overt growth deficiencies. Fourteen \textit{toa1} block mutants caused lethality or slow/Ts$^+$ growth (Table 1). All such \textit{toa1} mutants involve amino acids within either the N or C termini of Toa1, results consistent with a previous deletion analysis of \textit{TOA1} performed by Hahn and colleagues (56). Steady state protein levels were lower than WT for many of these \textit{toa1} mutants (not shown).

Similarly, a number of \textit{toa2} 2-Ala block mutations caused growth defects. Seventeen \textit{toa2} mutants resulted in lethality, whereas nine were slow growing and/or Ts$^+$. The mutated residues responsible spanned the length of Toa2 (Table 1). All \textit{toa2} mutants were readily detectable by immunoblotting (data not shown).

We followed these Ala block mutagenesis experiments by generating and testing the growth of yeast strains carrying a single Ala substitution of several Toa2 amino acids compared with the corresponding block mutants (Table 1). Finally, we identified those \textit{toa1} and \textit{toa2} mutants that compromised pro-
TABLE 1
toa1 and toa2 alanine block mutants that display growth deficiencies

| toa1 variants exhibiting slow/temperature-conditional growth |
|-------------------------------------------------------------|
| V17A/V18A/N19A                                              |
| L38A/K39A/N40A                                              |
| I41A/W42A/Q43A                                              |
| N242A/L243A/M244A                                           |
| L245A/C246A/L247A                                           |
| V251A/T252A/R253A                                           |
| R257A/W258A/K259A                                           |
| T275A/F276A/Q277A                                           |
| R8A/V9A/Y10A                                                |
| E11A/I12A/I13A                                              |
| L35A/Q36A/D37A                                              |
| C260A/S262A/L262A                                           |
| V266A/V267A/T268A                                           |
| I269A/N270A/R271A                                           |
| R38A/K39A/N40A                                              |
| I41A/W42A/Q43A                                              |
| N242A/L243A/M244A                                           |
| L245A/C246A/L247A                                           |
| V251A/T252A/R253A                                           |
| R257A/W258A/K259A                                           |
| T275A/F276A/Q277A                                           |

| toa2 lethal variants |
|----------------------|
| V10A/R11A            |
| D24A/T25A            |
| L26A/L27A            |
| E22A/E33A            |
| V40A/L41A            |
| E42A/T43A            |
| F44A/D45A            |
| L66A/D67A            |
| T68A/Y69A            |
| C72A/D73A            |
| D74A/V75A            |
| W76A/T77A            |
| F78A/I79A            |
| V80A/K81A            |
| Q84A/V85A            |
| K110A/L111A          |
| R112A/I113A          |
| S18A/L19A            |
| V20A/D21A            |
| M38A/R39A            |
| V62A/K63A            |
| G64A/N65A            |
| G70A/F71A            |
| T86A/V87A            |
| I106A/S107A          |
| C116A/N117A          |

Toa1 and Toa2 variants accumulate to levels that are similar to WT. Choosing the subset of Toa2 variants expected to minimize potential global affects of toa2 mutation by focusing on Ala mutations that alter Toa2 amino acids within the N-terminal domain four-helix bundle of the Toa1-Toa2 TFIIA heterodimer: amino acid residues Arg11, Ser18-Leu19, Asp24, Leu26, Ile27, Ile32, Met38-Arg39, Leu41, Phe44, and Asp65. All of these evolutionarily conserved, solvent-accessible residues are distinct from the Toa1-Toa2 dimerization domain and located far from the TBP-TATA DNA-binding portion of TFIIA (Fig. 3). Consequently, we expected that all functions of TFIIA containing these Toa2 variants would be WT for “basal” TFIIA functions.

We first showed both that recombinant Toa2 variants could efficiently assemble in vitro with recombinant WT Toa1 to form TFIIA and that the resulting TFIIA could be readily puri-
Toa2 Residues Contribute to TFIIA-TFIID Interaction through the RBD of Tafl4—Having identified RPG transcriptional defects in a variety of TFIIA mutants, particularly mutations in TOA2, and knowing that mutations in both TAF4 and TAF5 RBD-encoding sequences also cause defects in RPG transcription (24), we reasoned that compromised interaction between TFIIA and TFIID could account, at least in part, for this transcriptional phenotype. Further, because we had previously mapped the Tafl Rap1 binding domain to Tafl4 amino acids ~250–~350 (24), we speculated that binding of both Rap1 and TFIIA to the RBD would provide a potent and efficient regulatory node for RPG transcription-specific protein-protein interactions. Consequently, we checked whether TFIIA and TFIID interacted, and if so, whether this interaction depended upon Tafl4 RBD sequences. To test these hypotheses, we cultured pseudodiploid cells expressing TAF4 and the lethal variant TAF4Δ284–326. The taf4Δ284–326 allele carries a deletion of the portion of TAF4 encoding most of the Tafl RBD, amino acids 284–326. Both the WT and Taf4Δ284–326 forms of Tafl4 incorporate into TFIID (Fig. 5A), results consistent with the fact that there are 2 mol of the Taf4-Taf12 heterodimer per mol of TFIID (15, 44). Moreover, TFIID integrity, as scored by HA-Tafl4 and HA-Taf4Δ284–326 anti-HA IgG co-immunoprecipitation (co-IP) assays, is roughly equivalent regardless of whether WT or WT + Δ284–326 forms of Tafl4 were coexpressed (Fig. 5B). Importantly, anti-FLAG co-IP experiments using strains expressing the HA-tagged Tafl4 (WT or Δ284–326) and FLAG-tagged Toa2 show that TFIIA-TFIID coprecipitation is reduced by ~90% in the RBD-deleted Tafl4-expressing strain (Fig. 5C). These data suggest both that TFIIA and TFIID interact, perhaps via direct binding, and second, that this interaction is dependent upon the RBD of Tafl, as we had speculated.

In order to test whether TFIIA Toa2 sequences contribute to TFIIA-TFIID interaction, we prepared WCEs from toa2 Y10A-, toa2 F71A-, and toa2 Y10A/F71A-expressing cells and again scored TFIIA-TFIID interaction via co-IP. The WCE IP input shows essentially equal amounts of Toa2 and Tafl4 in all extracts (Fig. 5D, Lysate). Anti-HA-Toa2 TFIIA immunoprecipitates were formed using bead-bound anti-HA IgG, and the resulting immunoprecipitates were probed with either anti-HA IgG to score IP efficiency or with polyclonal rabbit anti-Tafl4 IgG to monitor the amounts of TFIIA-TFIID complex in the different strains. TFIIA containing the Ala-substituted Toa2 proteins showed reduced TFIIA-TFIID coprecipitation (from ~5 to ~50% of WT Toa2; see legend to Fig. 5). These data indicate that the TFIIA-TFIID interaction is dependent upon specific N-terminal Toa2 residues, a result consistent with binding of these two multisubunit complexes via specific contacts that require unique surfaces within subunits of both coactivators.

**FIGURE 2.** *toa1* and *toa2* Ts+ mutants exhibit reduced RPG transcript levels upon a shift to non-permissive temperature. A, steady state RNA levels in WT and *toa1* and *toa2* Ts+ mutants grown at 23°C or for 2 h at 37°C; see bottom of gel image. Shown is a representative K-screen image of gel-fractionated multiplex S’ primer extension products (24, 35); U3 RNA was the loading control. RNA from two independent TOA1 cultures and four independent WT TOA2 cultures were analyzed as controls. B, quantification of the RPS5 signals in A, adjusted for U3 content and normalized to the leftmost TOA1 or TOA2 RPS5/ U3 values; WT values = 100%. C, RNA-seq measurements of total RPG regulon RPKM from WT TOA2 (performed in duplicate and averaged; WT TOA2 total average RPKM = 278,970 ± 3101; error bar, S.E) and selected toa2 Ts+ mutants grown at 37°C; no error bars because *n* = 1. Data are normalized to the WT (TOA2 value = 100%). The primary RPKM data for the individual RPGs and selected transcription proteins are presented in supplemental Tables 1 and 2.
To more fully explore RBD/Toa2-mediated TFIID-TFIIA interactions, we tested whether overexpression of Taf4 or several other related transcription proteins might rescue the Ts+/H11001 growth of the toa2 conditional mutants. Such a genetic, high-copy suppression interaction(s) would support the notion of direct, physical TFIID-TFIIA Toa2 interaction mediated by Taf4. Synthetic gain- and loss-of-function genetic experiments have been widely used to support the possibility of direct interactions between proteins.

We performed growth assays with yeast expressing a single toa2 Ala mutant transformed with a multicopy plasmid encoding Toa2, Toa1, TBP (Spt15), Taf4, Taf11, or Rap1. All of these

FIGURE 3. Many toa2 Ala mutants change conserved residues that co-localize in TFIIA. A, alignments of S. cerevisiae (S.c.), Drosophila melanogaster (D.m.), and Homo sapiens (H.s.) Toa2 proteins. Amino acid identity is shown boxed in dark gray; similarity is boxed in light gray. Ala substitutions conferring Ts+ growth (red) or lethality (blue) are indicated. Protein structural elements (α-helix, β-sheet, and unstructured) are indicated and color-coded (orange, green, and teal, respectively). B, the location of mutated Toa2 residues on the TFIIA-TBP-TATA structure (70–72) (Protein Data Bank code 1RM1) are indicated by their amino acid sequence numbers.
TFIID-TFIIA-Rap1 Regulatory Interactions

A

B

C

D

E

TFIIA: TBP:

TATA-TBP-TFIIA

32p-TATA

R11A S18A-L19A D24A L26A

L26A

C

Rap1: TFIID: R11A L19A D24A L26A I7A I32A M38A R39A L41A F44A D45A

Fold Stimulation Transcription

Depleted +TIIA Depleted +TFIIA +Rap1 +TFIIA +Rap1

Transcription vs. WT

WT R11A S18-L19A D24A L26A I7A I32A M38A R39A L41A F44A D45A

In Vitro Transcription

In Vivo RPG Regulon Transcription

Transcription % WT

R11A S18-L19A D24A L26A I7A I32A M38A R39A L41A F44A D45A
genes encode proteins known to interact or suspected of interacting with Toa2/TFIIA. As expected, growth of all of the mutants was restored to WT levels by high copy Toa2 (and Toa1). By contrast, high copy SPT15 and RAPII slightly inhibit growth, even of the WT strain (Fig. 6). Growth of the toa2 D24A-expressing mutant at elevated temperatures was modestly improved by high copy TAF4, whereas growth of the L41A mutant was significantly increased by high copy TAF11 (Fig. 6).

Toa2 mutants R11A, I27A, I32A, and F44A behaved similarly to the D24A toa2 allele, whereas D45A toa2 growth patterns

**FIGURE 5.** TFIIA interacts with TFIIID through Taf4 RBD sequences 284–326, and interaction depends upon TFIIA Toa2 N-terminal domain sequences. A, WT Taf4 and the Δ284–326 Taf4 variant efficiently incorporate into TFIIID. Untagged, HA-tagged, or FLAG-tagged (indicated with plus or minus signs), WT Taf4 (WT) or the Δ284–326 (Δ) variant were coexpressed in yeast. WCEs were generated (Lysate, left) and used to prepare either anti-HA (middle) or anti-FLAG immunoprecipitates (right), see (labeled at the top). Immunoprecipitates were probed with anti-HA or anti-FLAG IgGs to score incorporation of the different forms of Taf4 in TFIIID. B, the incorporation of either WT Taf4 or the Δ284–326 Taf4 has no effect upon TFIIID integrity. TFIIID integrity was scored by anti-HA IP of Taf4 (WT or mutant) and co-IP of Taf5, Taf11, and Taf12. C, the binding of TFIIA to TFIIID is dramatically compromised for TFIIA containing the Δ284–326 Taf4 variant. TFIIA-TFIIID association was scored by anti-FLAG IP of FLAG-Toa2 and accompanying co-IP HA-Taf4 (HA-WT or mutant). Data shown are representative co-IP data. The corresponding x-ray films were scanned, and HA-Taf4/FLAG-Toa2 signals were analyzed using Bio-Rad Quantity One software to quantify the efficiency of TFIIA association with TFIIA in the different IP reactions. Taf4/TFIID-TFIIA co-IP efficiency, adjusted for IP efficiency of FLAG-Toa2 is as follows: Taf4 WT, 100%; Taf4 Δ284–326, 10.9%. We were not able to detect TFIIA in anti-Taf immunoprecipitates (not shown), presumably due to the small amount of TFIIA bound to TFIIID. D, TFIIA-TFIIID association scored by anti-HA-Toa2 co-IP in whole cell extracts of pseudodiploid cells expressing HA-Toa2 Y10A, HA-Toa2 F71A, HA-Toa2 Y10A/F71A, untagged WT Toa2, or HA-Toa2. Top (Lysate), WCE protein levels probed with anti-Taf4 (αTaf4) polyclonal IgG, or anti-HA IgG (αHA). Bottom (IP), protein levels in anti-HA-Toa2 immunoprecipitates as scored by immunoblotting immunoprecipitated proteins with anti-Taf4 (αTaf4) or anti-HA (αHA) IgGs. Data shown are representative co-IP data. The corresponding x-ray films were scanned, and HA-Toa2/HA-Toa2 signals were analyzed using Bio-Rad Quantity One software to quantify the efficiency of TFIIA association with TFIIA in the different IP reactions. IP efficiency-adjusted TFIIA-TFIIA association values are as follows: WT Toa2, 100%; Y10A Toa2, 52.9% WT; F71A Toa2, 20.3% WT; Y10A/F71A Toa2, 6.3%.

**FIGURE 4.** Most TFIIA variants exhibit WT basal activity but are variably deficient for Rap1-activated transcription *in vitro*. A, SYPRO Ruby-stained SDS-PAGE, indicating purity and concentration of purified recombinant TBP and reconstituted and purified recombinant TFIIAs. B, comparison of TFIIA variant relative specific activity in stabilization of TBP–TATA DNA complexes during EMSA. Representative K-screen image of EMSA reactions fractionated on native 6% SDS-PAGE, indicating purity and concentration of purified recombinant TBP and reconstituted and purified recombinant TFIIAs. C, comparison of TFIIA variant relative specific activity in stabilization of TBP–TATA DNA complexes during EMSA. Representative K-screen image of EMSA reactions fractionated on native 6% SDS-PAGE, indicating purity and concentration of purified recombinant TBP and reconstituted and purified recombinant TFIIAs. D, comparison of TFIIA variant relative specific activity in stabilization of TBP–TATA DNA complexes during EMSA. Representative K-screen image of EMSA reactions fractionated on native 6% SDS-PAGE, indicating purity and concentration of purified recombinant TBP and reconstituted and purified recombinant TFIIAs. E, comparison of the activities of WT and variant Toa2-TFIIA forms to support Rap1-driven RPG transcription in vivo (variously colored bars) and Rap1-driven transcription in vitro (red bars). Plotted are the total RPG regulon RPKM data (as a percentage of WT TFIIA) and the various TFIIA variants (indicated) along with the corresponding data for the efficiency of the same variant forms of TFIIA to support Rap1-activated transcription *in vitro* (as a percentage of WT TFIIA). The in vivo data are taken from supplemental Table 1 and Fig. 2C; in vitro data are taken from D.
resembled those of L41A in these overexpression plate growth tests (data not shown). Collectively, these synthetic, allele-specific positive and negative genetic interactions indicate likely links between TFIIA, Taf4, Taf11, TBP, and Rap1 and further support the hypothesis that specific toa2 mutants are defective in making direct, specific coregulatory interactions with TFIID. Additionally, these data again suggest a likely Taf target within the context of holo-TFIID-Taf4 (and perhaps Taf11; see Refs. 26 and 74).

Mapping Critical Domains within TAF4 RBD-encoding Sequences via Alanine-scanning Mutagenesis

To pursue the ideas that direct TFIIA-TFIID binding is mediated through direct Toa2-Taf4 RBD interactions and that these interactions are critical for RPG transcription, we subjected essential Taf4 RBD amino acids 250–350 (24) to alanine block scanning mutation. 6-Ala, 2-Ala, 1-Ala, and ultimately codon randomization mutations within DNA encoding Taf4 amino acids 251–346 were sequentially generated and screened. Each screen was guided by the data generated from the preceding screen (complete data set not shown). Only one 6-Ala block mutant, RBD amino acids 317–322, conferred a Tˢ growth phenotype. This Tˢ phenotype (Fig. 7) was at least as severe as any of our three previously described taf4 Tˢ mutants (taf4-116, taf4-141, and taf4-219 (24)) control strains as well as the growth of the Taf4 6-Ala mutant 317–322 (317–322), 2-Ala mutants G316A/W317A and W317A/L318A, or single mutants W317D and W317E.

Mapping Critical Domains within TAF4 RBD-Encoding Sequences via Alanine-scanning Mutagenesis

To reconcile how a single Taf4 residue might compromise multiple RBD functions, such as TFIIA-TFIID and TFIID-TFIIA-Rap1 Regulatory Interactions

FIGURE 6. Tˢ growth phenotypes of toa2 strains can be partially suppressed by overexpression of Tafs. Shown are high copy suppression analyses of toa2 D24A and L41A mutants in plasmid shuffle assays. Effect of 2μ vectors carrying the indicated genes (empty vector (−), TOA2, TOA1, SPT15/TBP, TAF4, TAF11, or RAP1, as shown on the left) on growth of cells expressing TOA2 alleleX, where X represents WT, toa2 D42A, or toa2 L41A allele. Growth was scored as detailed in the legend to Fig. 1.

FIGURE 7. Systematic Ala mutagenesis identifies Trp317 as a key Taf4 residue. Shown are the informative mutant strains identified by systematic, multistep Ala-scanning/codon randomization mutagenesis screens of the TAF4 RBD-encoding sequences (Taf4 amino acids 251–346). Plasmid shuffle was used to assess the growth of various Taf4 Ala-mutated strains. Shown are positive (TAF4) and negative (vector, taf4-250–350, taf4Δ284–326, taf4-116, taf4-141, and taf4-219 (24)) control strains as well as the growth of the Taf4 6-Ala mutant 317–322 (317–322), 2-Ala mutants G316A/W317A and W317A/L318A, or single mutants W317D and W317E.
Rap1-TFIID interactions, we speculated that additional Taf4 residues might contribute to these binding events. To address this hypothesis, we tested whether combining Taf4 Ala block mutants with the W317D variant would result in SSL growth phenotypes. Interestingly, every 6-Ala block mutation tested within the 251–286 interval, as well as the 306–310 block,
resulted in synthetic slow growth when combined with W317D (Fig. 8A).

Cells carrying five of the $taf4$ mutants displaying SSL growth phenotypes grew in liquid culture when the variant protein was the only form of Taf4 in the cell. These five yeast strains were utilized for co-IP experiments (Fig. 8B) to determine whether TFIID-TFIIA and/or TFIID-Rap1 binding was compromised in the synthetic variants. Although TFIID integrity was similar among the $taf4$ mutants (Fig. 8B, middle), the W317D substitution severely compromised Rap1-TFIID binding (Fig. 8B, middle); all Taf4 variant proteins were as stable as WT. Subsequent GST pull-down assays conducted with purified recombinant Rap1 and purified recombinant WT and mutant Taf4 proteins showed both that the binding of Rap1 with the TFIID subunit Taf4 is direct and that binding is sensitive to mutation of Taf4 tryptophan 317 to aspartic acid (Fig. 8C).

By contrast with Rap1-TFIID binding, TFIIA-TFIID binding was only modestly affected by the W317D substitution. However, when the W317D Taf4 mutation was combined with 269–274, 281–286, or 306–310 6-Ala block Taf4 mutations, a severe decrease in TFIID-TFIIA complex formation was observed (Fig. 8B, right). Collectively, these data indicate that multiple Taf4 RBD residues contribute to interaction with TFIIA. Moreover, the correlation between $taf4$ mutant slow growth and deficit in Rap1-TFIID and TFIIA-TFIID binding indicates that both of these events are functionally important.

To more precisely define the residues contributing to Taf4/TFIID-TFIIA binding, we combined W317D with the 2-Ala block and 1-Ala substitutions. The 2-Ala-W317D synthetic TFIIA-TFIIA binding, we combined W317D with the 2-Ala mutation with or without high copy TOAI + TOA2. Specific RPG mRNAs were less abundant in each of the $taf4$ mutants lacking high copy TFIIA compared with WT (Fig. 10B). TFIIA overexpression had no positive effect on RPG mRNA levels in WT cells, whereas RPG transcripts were increased in each of the $taf4$ mutants with high copy TOAI + TOA2. Quantification of RPS2 mRNA showed a roughly 2-fold increase in transcript levels in many of the $taf4$ strains when TFIIA was overexpressed (Fig. 10C); RPS3 and RPS5 mRNAs were similar (Fig. 10B) (data not shown). Once more, these data support the notion that TFIIA and TFIID directly interact through specific Toa2 and Taf4 RBD domains. Note that the TFIID-independent PGK1 mRNA was also increased when TFIIA was overexpressed; PGK1 is a known TFIIA-dependent gene (26, 74). We speculate that the effect of TFIIA overexpression on PGK1 transcription could be due to a general acceleration of cellular metabolic activity when TFIIA levels rise.

DISCUSSION

Activator-stimulated conformational rearrangements of Taf-containing complexes have been proposed based upon biochemical and functional studies of TFIID binding to enhancer-promoter DNA in the presence and absence of specific trans-activators (33, 34, 80 – 84). Moreover, single molecule studies of the effects of activators and TFIIA on TFIID-promoter DNA binding are consistent with such models, whereas recent EM studies confirm that structural transitions can be stimulated by TFIID-promoter DNA binding in the presence of TFIIA (85, 86). Although several laboratories have reported that TFIIA is not required in vitro for basal transcription catalyzed by purified RNA polymerase II plus TFIIE, -F, and -H and TBP (3), it is notable that two very recent studies (87, 88) have shown that TFIIA is essential for the formation of cryo-EM-visualizable, functional PICs formed from purified components. Finally, quaternary complexes of Rap1 enhancer-promoter
DNA, TFIIA, TFIID, and Rap1 undergo induced conformational rearrangements (42). Thus, several groups agree that these key transcription proteins can interact dynamically upon engaging enhancer-promoter DNA. However, neither the physiological relevance of such activator-induced TFIID and TFIIA conformational rearrangements toward PIC formation in vivo nor the exact TFIID or Taf domain(s) involved are known. This lack of physiological data was the driving force for our embarking on the series of studies described in this report. We believe that analysis of yeast RPG transcription affords

**FIGURE 9.** SSL growth tests identify multiple Taf4 residues that collaborate with Trp^{317} to support growth and facilitate Taf4-TFIIA interaction. A, plasmid shuffle growth assays of strains carrying the indicated form of TAF4 (left): empty vector, WT TAF4, W317D, or 1-Ala_{x}/H11001 W317D combinations. B, left, effect of Taf4 amino acid substitutions (W317D and R308A/W317D) on direct Taf4-TFIIA interaction. Purified MBP (8000 fmol) or MBP-Taf4/12 heterodimer variants (4000 fmol) were bound to amylose-Sepharose beads and incubated with increasing amounts of purified TFIIA. Binding (white arrows; Toa1 (top) and Toa2 (bottom)) was scored by SDS-PAGE as in Fig. 8 (left); background binding to MBP alone was subtracted from all binding reactions. Right, quantification of binding data. C, binding assays were repeated for WT and R308A/W317D Taf4 variants as in B, except that the binding reactions were performed with five replicate binding reactions for each TFIIA concentration. The data were quantified and plotted (open circles, WT; filled triangles, R308A/W317D); background binding to MBP alone was subtracted from all binding reactions; the 95% confidence intervals for the plots are shown for each curve (dashed lines), as are the mean and S.E. (error bars) for each individual measurement. t tests indicated that the R308A/W317D Taf4 variant bound 2-fold less TFIIA than WT Taf4 at all concentrations tested (p < 0.05).
TFIID-TFIIA-Rap1 Regulatory Interactions

unique opportunities for systematically addressing these gaps in our understanding of the mechanism of action of the TFIID and TFIIA coactivators.

The vigorously transcribed RPGs are TFIID-dependent, and transcription of this gene family is driven in large part through direct Rap1-Taf interactions (24, 35). Moreover, mutations that weaken Rap1-TFIID (Taf4/5) RPG transcription-activating contacts exhibit overt growth deficiencies (24). This is due to the fact that yeast cell growth is so tightly linked to expression of the genes encoding the components of the translation machinery (i.e. RPGs). We capitalized upon this relationship between RPG transcription and cellular growth to systematically probe the importance of individual TFIID residues to RPG transcription, initially by monitoring yeast cell viability and temperature-conditional growth.

Consistent with earlier reports (26, 27, 55, 56, 79), we found the TOA1 and TOA2 are sensitive to Ala substitutions (Fig. 1 and Table 1). This was particularly true for TOA2. Toa1 apparently makes more significant contributions to TFIID complex formation/stability because a large portion of our toa1 Ala mutants destabilized Toa1 and hence TFIID levels. Consistent with the idea that Toa2 N-terminal residues make critical protein-protein interactions with coregulators like TFIID, we identified multiple toa2 Ala substitutions with WT protein stability that induced slow growth (Fig. 1). Several of our mutants are consistent with previously reported toa2 mutants that affect N-terminal residues Asp\(^{24}\) and Ile\(^{27}\) (26, 27, 56). The clustering of many of these Ala-substituted residues within the TFIID/Toa2 structure suggests shared function(s) (Fig. 3).

To assess the possible contributions of Ala-mutated Toa1 and Toa2 residues toward ribosomal protein gene expression, we tested RPG transcription at permissive and non-permissive temperatures in a subset of our mutant collection. These analyses confirmed the importance of TFIID function to transcription of both individually analyzed RPG mRNA-encoding genes (Fig. 2, A and B) and the entire RPG regulon in vivo (Fig. 2C). Results from the use of these same mutants for in vitro studies, such as TBP-TATA DNA complex stabilization and transcription (Fig. 4, B–D), suggest quite similar allele-specific patterns of Toa2 residue function with RPG regulon transcription (Fig. 4E), although some differences were evident between in vivo RPG mRNA results and the biochemical data. For example, toa2 122A exhibited the most severe RPG transcript phenotype of all of the mutants in vivo but not in vitro (Figs. 2 and 4). Similarly, the toa2 111A in vivo data did not correlate perfectly with the biochemical data. Overall, however, considering that these are quite different assays (i.e. one assay scored expression of all RPGs (Fig. 2C), another scored the in vivo transcription of a handful of RPGs (e.g. Fig. 2, A and B; RPS2, RPS3, and RPS5), and the third scored the in vitro transcription of a simple chimeric TFIID-dependent, Rap1-driven synthetic reporter gene (24, 35) (Fig. 4D)), we believe it is highly significant that these results are so similar regarding the relative contribution of individual TFIID residues to RPG/Rap1-driven transcription. We also observed toa2 allele-specific effects upon TFIID-TFIID interaction (Fig. 5). Ala mutation of N-terminal sequences located within and abutting the TFIID four-helix bundle (Fig. 3B) affected TFIID-TFIID binding.

Consistent with previous reports of direct binding of purified TFIID and Taf4 (28, 29), we obtained genetic evidence suggesting TFIID-TFIID interaction through Taf4 and Toa2 D24 (Fig. 6). Notably, the toa2 D24A mutant exhibits a strong Ts\(^{+}\) growth phenotype, normal protein abundance, and severely compromised RPG transcription in vivo (Figs. 1 and 2). TFIID containing Toa2 D24A efficiently binds and stabilizes the TBP-TATA DNA complex yet has the weakest ability to reconstitute in vitro transcription, whether Rap1-TFIID- or Gal4-VP16/ SAGA-dependent (Fig. 4). Hahn and colleagues (27) have previously described cross-linking of TFIID to Taf4 and Spt8 in vitro, results suggesting that TFIID-TFIID and TFIID-SAGA interactions occur through similar TFIID N-terminal residues, including those proximal to Toa2 Asp\(^{24}\). These workers also observed conditional growth of toa2 D24A mutants and SSL genetic interactions between toa2 D24L and both spt8 and spt3 null alleles (27). Thus, it is likely that TFIID provides coactivator functions on both TFIID-dependent and SAGA-dependent genes by using Toa2 Asp\(^{24}\) as a component of distinct protein-protein interaction pathways. RPG transcription in vivo is TFIID-dependent but SAGA-independent (4, 24, 35, 89). Notably, in addition to our reported binary TFIID-Taf4 interaction, Stargell and colleagues (26, 74) have identified and analyzed TFIID-Taf11 binding through Toa2 Ile\(^{27}\). We too observed genetic interactions between TOA2 and TAF11, but through Toa2 residue Leu\(^{41}\) rather than Ile\(^{27}\) (Fig. 6). The reason(s) for this discrepancy is unclear at present. As with most genetic interaction studies, overexpression complementation of the type we observed here with TAF4 and/or TAF11 can only support the idea of direct protein-protein interactions. In this report, these experiments represent but one form of data supporting direct Taf2/TFIID-Taf4/TFIID binding.

Given the apparent evolutionary conservation of the putative TFIID-TFIID interaction via Taf4, we pursued characterization of the TFIID binding domain in Taf4. Preliminary analyses (Fig. 5, A–C) implicated Taf4 residues (24) as the domain of Taf4 that formed the binding site for TFIID. The possibility that the Taf4 RBD, the direct binding site for Rap1, was also a binding site for TFIID was intriguing. If multiple regulatory protein interactions targeted the Taf4 RBD, such multivalent interactions would provide important new insights into the physiological relevance and mechanism underlying the formation of the stable, conformationally rearranged Rap1-TFIID-TFIID-DNA quaternary complex that we have observed previously (42).

We performed a series of genetic screens of the RBD-encoding sequences of TAF4 and found that Taf4 amino acid residue

FIGURE 10. Overexpression of TFIID rescues the synthetic growth and RPG transcription deficiencies of toa1-1-Ala, \(+\)W317D mutants. A, plasmid shuffle assays tested the effect of high copy TOA1 + TOA2 on the growth phenotypes of TAF4 lethal toa1 toa2+ strain, W317D, or the indicated Taf4 1-Ala, +W317D strains. Only the TOA1-Toa2 3′ to 3′ gene orientation efficiently rescued growth (data not shown). B, total RNA was prepared from room temperature cultures of shuffled mutant strains with or without high copy TOA1/TOA2 and subjected to multiplex 5′ primer extension analysis as in Fig. 2. A representative K-screen gel scan image is shown. C, quantification of RPS2 mRNA levels.
Trp$^{317}$ was the most important single residue within the mapped TFIIA-RBD interaction network (Fig. 7). However, additional genetic screens of the Taf4 RBD demonstrate that there are clearly additional amino acid residues that collaborate with Trp$^{317}$ (Fig. 8). Combining Taf4 RBD Ala block mutations with W317D resulted in SSL and Ts$^{+}$ growth defects that correlated with weaker TFIIA-TFIID interaction than that seen with Taf4 W317D alone (Fig. 8B). W317D reduced but did not eliminate apparent Rap1-Taf4 binding, consistent with polyclonal Rap1-TFIID interactions occurring through Taf4, Taf5, and Taf12 (24); this was evident in direct Rap1-Taf4 binding assays with purified recombinant proteins (Fig. 8C). Moreover, the loss of function in both TFIIA-Taf4 and Rap1-Taf4 binding that resulted from W317D indicates likely overlapping functions of Taf4 residues that direct each binding event. Our additional Ala substitution mutagenesis and SSL screens with TAF4 identified the responsible Taf4 residues as Leu$^{259}$, Arg$^{260}$, Glu$^{271}$, Arg$^{272}$, Arg$^{277}$, Ile$^{278}$, Ala$^{279}$, Leu$^{280}$, Gly$^{281}$, Leu$^{282}$, Arg$^{308}$, Ala$^{309}$, and Ser$^{311}$. All of these amino acid residues about Trp$^{317}$ (Fig. 9A). Structural studies could determine whether these residues actually reside within close proximity in the tertiary structure of Taf4. Consistent with the overlapping function model, the R308A/W317D variant protein displays markedly reduced direct binding to TFIIA in vitro (Fig. 9, B and C). Thus, it is likely that these Taf4 RBD residues contribute to both TFIIA-TFIID binding and Rap1-TFIID binding, although the determination of the extent of functional overlap must await future fine structure mapping.

Given these observations, we hypothesized that overexpression of TFIIA might rescue both the SSL/Ts$^{+}$ growth phenotypes of the taf4 Ala$^{+}$/W317D mutants and the observed RPG transcription defects if slow growth was indeed caused by deficiencies in TFIIA-TFIID and Rap1-TFIID interactions. As a control, we tested the taf4D$^{284-326}$-expressing strain in this assay. These cells carry a mutation that removes much of the RBD and, consequently, the TFIIA/Rap1 binding residues mapped herein. The taf4D$^{284-326}$-derived strains are inviable, despite the fact that the expressed, truncated Taf4 protein is stable (24) and incorporates efficiently into TFIID (Fig. 5A). Not surprisingly, overexpression of TFIIA does not rescue growth of this strain (Fig. 10A), indicating that TFIIA overexpression per se cannot bypass the requirement for TFIIA- and/or Rap1-TFIID interactions. By contrast, overexpression of TFIIA corrected the SSL/Ts$^{+}$ growth of both the single W317D mutant and the synthetic Ala$^{+}$/W317D mutants (Fig. 10A); notably, even the nearly inviable R308A/W317D mutant grew well when TFIIA was overexpressed. Finally, and most importantly, analysis of RPG mRNA gene transcription in these same strains showed that overexpression of TFIIA restored mRNAs to nearly WT levels (Fig. 10, B and C), providing strong support for our hypotheses regarding TFIIA-/Rap1-TFIID binding and RPG expression. Presumably, elevated TFIIA increases binding and/or stabilizes the interaction of both TFIIA and Rap1 to the RBD of Taf4/TFIID, although it remains for these hypotheses to be tested directly.

The implications of our findings are that TFIIA and Rap1 both bind to TFIID through a small set of amino acids within the Taf4 RBD (as well as to Taf5 and Taf12 RBDs) (24, 35). We do not yet know the exact extent of overlap of the Taf4 residues used for Rap1 or TFIIA binding, nor do we know if binding occurs (see Fig. 11) simultaneously, cooperatively, or competitively with each other or with a possible regulatory repressor protein(s) (see below). Cryo-EM has shown (42) that in quaternary pre-PIC subcomplexes, Rap1 and TFIIA co-occupy a region of TFIID that contains one of the two asymmetrically exposed Taf4/Taf12 heterodimers present in the complex (40–44). Should competitive binding to TFIID occur, Rap1 might actively displace or restrict TFIIA to a different location on TFIID. Such a scenario is similar to the reported competition between the Spt8 SAGA subunit and DNA for TFIIA/TBP binding (27, 90) while also reminiscent of the model proposed by Olave et al. (29), wherein the mammalian RBP repressor displaces human TFIIA by competition for a common binding site on human Taf4. In such models, a TFIIA-Rap1 competitive dynamic for binding to the Taf4 RBD might help to establish alternate modes of TFIID-promoter DNA binding involving TFIIA-TBP interactions and/or Rap1-TFIID-DNA binding, thereby initiating key conformational alterations of the Rap1-TFIIA-TFIID-DNA complex that are important for stabilizing this assembly.

Final tests of these ideas will require the generation of true separation-of-function mutant alleles of TAF4, RAP1, and TOA2. Preferably, such separation-of-function mutants will be tested in the context of intact, purified TFIIA, Rap1, and most importantly TFIIA, as was recently reported for the analysis of the contributions of the human Taf3 plant homeodomain finger domain in human TFIID transcriptional functions (91). The
tools and methods we have developed in this study, along with our efficient TFIID isolation protocols (15, 35), will greatly facilitate this endeavor. The utilization of such mutant TFIID for combined biochemical and structural studies will help to precisely define the mechanism(s) underlying PIC structural transitions and their contribution to transcription regulation.

Acknowledgments—We thank our colleagues Drs. Manish K. Tripathi and Iuliana M. Gilchuk for providing reagents and advice; Amanda Meyer for generating the PyMOL image presented in Fig. 3B; and Dr. Travis A. Clark for RNA-Seq analyses. J. H. L. thanks D. F. B. Miller and Dr. T. C. Kaufman (Indiana University, Bloomington, IN) for sharing their passion for molecular cloning and Dr. H. P. Erickson (Duke University) for a generous gift.

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