The Transcriptional Repressor Activator Protein Rap1p Is a Direct Regulator of TATA-binding Protein*\textsuperscript{1,5}

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Essentially all nuclear eukaryotic gene transcription depends upon the function of the transcription factor TATA-binding protein (TBP). Here we show that the abundant, multifunctional DNA binding transcription factor repressor activator protein Rap1p interacts directly with TBP. TBP-Rap1p binding occurs efficiently \textit{in vivo} at physiological expression levels, and \textit{in vitro} analyses confirm that this is a direct interaction. The DNA binding domains of the two proteins mediate interaction between TBP and Rap1p. TBP-Rap1p complex formation inhibits TBP binding to TATA promoter DNA. Alterations in either Rap1p or TBP levels modulate mRNA gene transcription \textit{in vivo}. We propose that Rap1p represents a heretofore unrecognized regulator of TBP.

Eukaryotic gene transcription is controlled through the concerted action of DNA-binding transactors, proteins that functionally interact with the transcription machinery to turn genes on and off. For mRNA-encoding genes the transcription machinery is composed of the general transcription factors (GTFs)\textsuperscript{2} TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase (RNAP) II. DNA-bound transactivators stimulate the GTFs and RNAP II to form pre-initiation complexes (PIC) on cis-linked promoters (1). TBP recruitment to the TATA box of mRNA gene promoters is a critical, and likely rate-limiting step in nucleating this process (2–4). TBP is chaperoned to promoters by a number of proteins, and in the case of mRNA-encoding genes, TFIID is the predominant protein complex that serves this function. TFIID has 15 evolutionarily conserved subunits, TBP and 14 TBP-associated factors (TAFs) (5, 6). The TATA box promoter element of mRNA-encoding genes is bound via the TBP subunit of TFIID through a process facilitated by TAFs (7–10).

TBP is required for nearly all nuclear gene transcription as it is an integral subunit of the RNAP I, II, and III-specific initiation factors SL1, TFIID, and TFIIB (11). Consequently, it is not surprising that TBP function is subject to tight regulation by non-GTF transcription factors such as NC2, Mot1p, and SAGA and NOT that all directly bind TBP and modulate its activity (12, 13).

Yeast Rap1p is encoded by a single-copy essential gene and plays multiple roles \textit{in vivo} (14–17). Rap1p is a key transactivator of over 300 co-regulated genes, including the ribosomal protein (RP) (18) and glycolytic enzyme-encoding genes (19, 20), and it drives 40% of all mRNA gene transcription initiation events in actively growing cells (18). Additionally, Rap1p participates in transcriptional repression (21), telomere length modulation (22), recombination (23), and chromatin barrier function (24, 25).

The only essential domain of Rap1p is its DNA-binding domain (DBD), which is composed of two Myb-like motifs. Yeast expressing only the Rap1p DBD grow, albeit extremely slowly (26). Deletion of the N-terminal BRCT-DNA bending domain has minimal effects on viability (27, 28), whereas removal of sequences C-terminal to the DBD results in slow growth (26). A C-terminal activation domain, fused to a heterologous DBD, weakly activates transcription of a reporter gene (29); by contrast, lexA full-length Rap1p fusions fail to activate (30). The C-terminal silencing domain represses mating-type, telomere-proximal, and RP genes under certain circumstances (31, 32). Finally, a 34-amino acid (aa) Tox domain inhibits cell growth when overexpressed fused to the DBD (17, 33–35).

Several mechanisms have been proposed for how Rap1p controls gene transcription. First, Rap1p-upstream activating sequence/enhancer (\textit{UAS}\textit{RAP1}) binding may exclude nucleosomes and stimulate transcription by increasing local DNA cis-element accessibility (30, 36). Rap1p binds nucleosomal \textit{RAP1} sites efficiently \textit{in vitro} (37), and \textit{in vivo} \textit{UAS}\textit{RAP1}, DNA is deficient in nucleosomes (30, 38–42) although some nucleosomal \textit{RAP1} sites are only bound conditionally (43, 44). Second, Rap1p functionally interacts with other DNA binding factors (\textit{i.e.} Gcr1p-Gcr2p, Afb1p, Reb1p, Fhl1p-Ihf1p, Sfp1p, and Hmo1p) (45–52), and with multисubunit transcriptional coregulators such as NuA4/Esa1p, SWI/SNF, and TFIID (34, 53, 54). We have recently shown that Rap1p directly binds several TAF subunits of TFIID (55). Third, Rap1p interacts with Rif and Sir proteins, which contribute to transcriptional repression of telomere-proximal and \textit{HML}/\textit{HMR} genes, as well as telomere-
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length modulation (31, 32, 56, 57). Despite the fact that Rap1p has been studied extensively, the exact molecular mechanisms through which these myriad Rap1p protein-protein interactions lead to transcription activation and repression remain to be deciphered.

In this report we describe the results of our continuing investigations into how Rap1p interacts with the transcription machinery. We have found that Rap1p directly and specifically binds TBP, and have molecularly dissected this interaction. We discuss the implications of Rap1p-TBP binding *vis à vis* the regulation of TBP activity.

MATERIALS AND METHODS

**Plasmids, Protein Purification, Yeast Strains, Yeast Cell Growth, and Manipulation**—For TBP, His<sub>6</sub>-TBP (58, 59) was used; other TBP variants were cloned in pBG101 as BamHI-EcoRI inserts for the production of His<sub>6</sub>-glutathione S-transferase (GST)-TBP and TBP. Rap1p variants were cloned in pET42a as EcoRI-XhoI inserts for the production of GST-Rap1p; His<sub>6</sub>-GST-Rap1p and Rap1p variants were produced in pET42a as EcoRI-XhoI inserts for the production of GST-Rap1p; His<sub>6</sub>-GST-Rap1p and Rap1p variants were produced from Rap1p forms cloned in pGBM1 (a version of pBG101 with BamHI and EcoRI sites inverted) as EcoRI-Xhol inserts. For TFIIA, pToa1 and pToa2 (60) were used; for TFIIIB, His<sub>6</sub>-tagged (61); for NC2, His<sub>6</sub>-tagged Ncb1p and Ncb2p (62); and for Tat1p TAND (TAF1 N-terminal domain), the TAF1 open reading frame sequence encoding amino acids (aa) 1–380 was PCR-amplified with NheI (5) and EcoRI (3) as an XhoI-flanked insert for the construction of pBG101-Rap1p, TAP-Rap1p, W303a (Rap1p-TAP); TAP-RAP1, W303a (RAP1-TAP::HIS3). MYC-tagged WT, His<sub>6</sub>-TBP, His<sub>6</sub>-TBP, and NC2 proteins were produced as described as listed above. His<sub>6</sub>-GST-TBP and His<sub>6</sub>-GST-Rap1p variants were expressed and purified for the production of GST fusion proteins. His<sub>6</sub>-GST fusion variants were bound to glutathione beads, glutathione-eluted, and treated with His<sub>6</sub>-tagged precission protease on Ni-nitriloacetic acid agarose beads for 1 h at room temperature and centrifuged to remove the beads. Supernatants were collected, analyzed for protein, and stored at −80 °C.

Tandem affinity purification (TAP)-tagged wild type (WT), W303a (MATa ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100 ssd1-1) TAP-Rap1p, W303a (TAP-RAP1::TRP1); Rap1p-TAP, W303a (Rap1p-TAP::HIS3). MYC-tagged WT, W303a (MATa ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100 ssd1-1); MYC-Rap1p, W303a (RAP1-MYC::TRP1) were used. Rap1p knockdown, control strain, ZMY60 (MATa ura3-52 trp1-Δ1 ade2-101 pACE1-UBR1 pACE1-ROX1) (63); Rap1p knockdown strain LEV391 ZMY60(rap1(Δ::KAN′) (KAN′-ANB-UB-R-lacl-4HA-RAP1) (64) were also used. RAP1 variants were cloned as EcoRI-ClaI inserts either in pESC vectors (UAS<sub>Gal4</sub>-dependent expression), in pRS415 vector (ADH1-enhancer/promoter) for the production of Rap1p-FLAG forms, or pRS414, controlled by RAPI enhancer/promoter and RAP1 terminator. Wild type TBP was cloned in the same pESC vector ± RAP1 as an Xhol-flanked insert for the expression of MYC-TBP (Fig. 5, B and C); TBP variants (Fig. 6) were cloned as BamHI-Xhol inserts in pRS413 (ADH1-dependent expression) for the production of HA<sub>3</sub>-TBP. Yeast were grown, transformed, plated, and subjected to 5-fluoroorotic acid plasmid shuffle growth tests using standard methods.

**DNase I Footprinting**—DNase I footprinting using Ad2 MLP (major late promoter) (−250 to +195 relative to the +1 transcription start site) and RPS1A (−42 to +23 relative to the A<sub>1</sub>TG Rps1p methionine codon) was performed as described (5).

**GST Pulldown Assay**—100-μl binding reactions were performed in binding buffer: 20 mM HEPES-KOH (pH 7.6), 10% glycerol, 70 mM potassium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM MgAc, 0.01% Nonidet P-40, plus 10 μl of beads (300 ng of GST fusion protein/reaction), and the proteins to be tested for binding. Reactions were mixed 30 min at room temperature, then beads were washed 3 times with binding buffer, resuspended in 20 μl of SDS-PAGE sample buffer, heat denatured, and analyzed by SDS-PAGE on NuPAGE 4–12% polyacrylamide gels followed by Sypro Ruby staining or immunoblotting.

**TBP Cross-linking**—TBP (100 ng/μl) in 19 μl of 20 mM HEPES-KOH (pH 7.6), 10% glycerol, 150 mM NaAc, 5 mM MgAc, and 0.05% Nonidet P-40 was incubated for 30 min at room temperature, then 1 μl of 20% formaldehyde, 20 mM HEPES-KOH (pH 7.6), 150 mM sodium acetate was added and incubation continued for 15 min at room temperature. Cross-linking was quenched by addition of 3 μl of 2.5 M glycine and incubation for 5 min at room temperature. 20 μl of SDS-PAGE sample buffer was added and samples were loaded, without heating, on 4–12% NuPAGE gels followed by Sypro Ruby staining or immunoblotting.

**Whole Cell Extract (WCE) Preparation, Immunoprecipitations (IPs), and Immunoblotting**—WCE preparation, IPs, immunoblotting, and chemiluminescent detection were performed as described (65).

**β-Galactosidase Assay**—Freshly transformed cells were used. Colonies were used to inoculate 5 ml of selective media cultures, grown to 1 A<sub>600</sub>/ml, and cells tested for β-galactosidase activity as described by Blair and Cullen (72).

**Primer Extension and Chromatin Immunoprecipitation (ChIP) Assays**—Primer extension (67) and ChIP assays were performed essentially as described (55) except ChIP PCR products were fractionated by non-denaturing polyacrylamide gel electrophoresis in 1× TBE buffer and DNA quantified by staining with Sypro Gold (Invitrogen).

RESULTS

While conducting footprinting experiments to examine the binding of yeast TFIIID to a battery of Rap1p-dependent and Rap1p-independent genes, control reactions showed that Rap1p addition blocked TBP-TATA DNA binding. Given the central role of TBP in nuclear gene transcription, the potency of inhibition of TBP-DNA binding by nanomolar concentrations of Rap1p, the nuclear abundance, and high concentration of both proteins (∼100 μM), we examined this phenomenon in more detail.

**Rap1p Inhibits TATA DNA Binding by TBP**—The inhibitory effect of Rap1p upon the binding of TBP (see Fig. 1A for purity of proteins) to the Adenovirus 2 major late promoter (Ad2 MLP), a heterologous DNA devoid of RAPI sites, is shown in Fig. 1B (left panel, Ad2 MLP). As expected, TBP bound to the Ad2 MLP TATA box (*lane 1 versus 4*), and at the TBP concen-
termination used here, also bound to two TA-rich regions up- and downstream of TATA (TA-I, TA-II) (5, 66, 67). Whereas Rap1p did not bind to the Ad2 MLP (lane 3), TBP binding at all three sites was potently inhibited when Rap1p and TBP were added simultaneously (lane 4 versus 5). However, either pre-binding of TBP to DNA (lane 9) or TFIIA addition (lane 7) protected TBP from the negative action of Rap1p. Similar results were observed with RPS1A DNA, a yeast gene containing consensus (RAP1) and non-consensus (RAP1*) Rap1p binding sites (Fig. 1B, right, RPS1A). In this case, Rap1p both bound to RAP1 sites (lane 13) and blocked TBP-DNA binding to RPS1A TA-rich sequences (TA-I, -II, -III, -IV; lane 15). Again, TFIIA addition (lane 17) and TBP-DNA complex formation (lane 19) prevented Rap1p inhibition. The extent of inhibition of TBP-DNA binding by Rap1p was dependent on the concentration of both proteins, consistent with a direct, bimolecular Rap1p-TBP interaction (Fig. 1C, compare lanes 5–9, 10–14, and 15–19).

Notably, TFIIA failed to block Rap1p from inhibiting TBP-TATA binding (Fig. 1D, compare lanes 20–22 with lanes 2–19 and 23–25). (Note that the apparent protection of DNA observed when both TBP and TFIIA are added to the footprinting assays is not reproducible (data not shown), and likely results from enhanced nuclease digestion of the probe when these two proteins are added simultaneously.) Together, these data suggested that Rap1p and TBP directly interacted, and that a specific domain(s) of TBP was targeted.

Mapping Rap1p Domains That Inhibit TATA DNA Interaction through Direct TBP Binding—To map the TBP inhibitory region(s), we generated a family of Rap1p variants (Fig. 2, A and B) and assessed their ability to prevent TBP binding to Ad2 MLP (Fig. 2C, lanes 3–12) and RPS1A (Fig. 2C, lanes 26–35). The Rap1p DBD specifically (Fig. 2C, lanes 36–46 versus...
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13–23) inhibited TBP-DNA binding to the TATA regions of both DNAs (Fig. 2C, compare lanes 5, 6, 8, 10, 12, and 28, 29, 31, 33, 35 versus lanes 3, 4, 7, 9, 11, and 26, 27, 30, 32, 34). To test whether the Rap1p DBD directly bound TBP we performed GST-Rap1p pulldowns. We found that all GST-Rap1p variants that contained the Rap1p DBD specifically bound TBP (Fig. 2D, lanes 7, 9, 16, 21, 25 versus 12, 14, 18, 23, 27). Collectively these data demonstrated that the Rap1p DBD interacted with TBP directly, and suggested a molecular explanation for our observation that Rap1p blocked TBP-TATA binding.

Mapping TBP Surfaces That Interact with Rap1p—To map the portions of TBP that interacted with Rap1p we performed pulldown competition assays to test whether well studied TBP-binding partners (TATA DNA, TFIIA, TFIIIB, NC2 (Ncb1p and Ncb2p) and the Taf1p TAND; Fig. 3A) inhibited TBP-Rap1p interaction. As expected from our previous work (58, 59, 68), all of the purified proteins (Fig. 3B) avidly bound TBP (Fig. 3C, lane 1 versus 2–6), indicating that these proteins were all highly active.

When added along with TBP to GST-Rap1p binding reactions, neither TFIIIB nor Ncb1p efficiently blocked TBP-Rap1p binding. By contrast, TFIIA, the NC2 Ncb2p subunit, and the Taf1p TAND all were able to block TBP-Rap1p complex formation quite efficiently (Fig. 3D, compare control lane 4, with lanes 7, 16, 19 versus lanes 10, 13). Similarly, double stranded WT (GGCTATAAAAGGGG), but not double stranded mutant (GGCTAAGAAAGGGG) Ad2 MLP TATA DNA prevented TBP-Rap1p complex formation (Fig. 3D, lane 21 versus 23). (We assume that neither Ncb1p nor TFIIB were GST-Rap1p-TBP associated due to a shorter half-life of these TBP complexes.) These data indicated that the DNA, NC2, TAND, and TFIIA interaction domains of TBP contributed importantly to Rap1p-TBP binding.

Identification of TBP Residues That Interact with Rap1p—To more precisely map the Rap1p interaction domain we used a family of truncation and point mutant forms of TBP (Fig. 4A). To document the published activities of these TBP mutants (Fig. 4B), and to demonstrate that all variants had some definable biochemical activity and hence were not inactive in Rap1p binding due to the fact that they were, for example, just unfolded, we measured both dimerization (Fig. 4B, lanes 2–19, X-linked, anti-TBP IgG I-BLOT) and DNA binding, ± Rap1p and ± TFIIA (Fig. 4C). As reported (69, 70), these TBPDs variably dimerized (Fig. 4B), and only TBP point mutant variants
E186Q, Q68R, F177R, G180R, and N95R bound TATA. In every case when DNA binding was observed TATA binding was sensitive to Rap1p and protected by TFIIA (Fig. 4C).

When tested via GST-TBP pulldown, all TBP point mutant variants except R98E and R196E bound Rap1p (Fig. 4D, cf. lanes 21 and 41). TBP amino acids Arg-98 and Arg-196 reside on opposite sides of the cavity forming the concave DBD of TBP (red shaded residues, Figs. 3A and 4A). Consistent with these data, only the GST-TBP truncation mutant composed of aa 71 to 240 was able to bind Rap1p (Fig. 4D, lane 52). These data indicated that when Rap1p bound TBP via (minimally) amino acids Arg-98 and Arg-196, access to the TBP DBD and hence DNA binding was blocked.

Rap1p and TBP Form a Complex in Vivo—To assess whether Rap1p and TBP interacted in vivo we performed co-immunoprecipitation (co-IP) studies. Whole cell extracts were prepared from control and tagged strains (TAP-, Myc-, HA-) that express Rap1p from the chromosomal RAPI promoter. WCEs were fractionated by SDS-PAGE, blotted, and probed with appropriate antibodies to verify the specificity of tagging, WCE TBP content, and equal protein transfer (Fig. 5A, left panels). The WCEs were also used to form IPs using anti-TAG antibodies. IPs were probed by immunoblotting with anti-Rap1p and anti-TBP IgGs to monitor co-precipitation (Fig. 5A, right panel, top and bottom). The results indicated that under normal physiological expression conditions Rap1p and TBP were indeed associated.

To test the ability of WT and mutant Rap1p variants to bind endogenous and exogenous TBP we used cells that expressed a tetracycline (TET)-regulated chromosomal RAPI allele (71) into which we introduced multicopy plasmids overexpressing Rap1p-FLAG and/or Myc-TBP under LIAS_GAL control (see schematic, Fig. 5B). Cells were grown on raffinose, +/− TET, and expression of the episomal TBP and Rap1p variants induced for 3 h by the addition of galactose. Under these conditions Rap1p-FLAG was able to interact with both endogenous and exogenous Myc-TBP (Fig. 5C, lanes 3, 4, 9, 10, 13, 14, 17, and 18; left panel, − TET, + TET), and, consistent with the biochemical analyses presented above, complex formation was Rap1p DBD-dependent. Similar results were observed in the reciprocal experiment (Fig. 5C, right panel). Together these data indicated both that Rap1p and TBP indeed interacted in

**FIGURE 3. Mapping TBP surfaces that contribute to Rap1p-TBP interaction.** A, three-dimensional ribbon structures of various TBP complexes; red-shaded residues are defined in the legend to Fig. 4A, B, Sypro Ruby-stained SDS-PAGE of 200 ng of the indicated purified proteins; MW, molecular weight markers. C, GST-TBP-loaded glutathione beads (300 ng of GST-TBP/reaction) were incubated with 300 ng of the indicated proteins (cf. lanes 1–6). GST-Rap1p (300 ng; mw 30 000) was added to each reaction, and was bound to GST-TBP-loaded glutathione beads in the presence of a 5-fold molar excess of TBP binding competitors, as indicated. GST-Rap1p (arrow) and bound TBP (black circles) are indicated. D, Sypro Ruby-stained SDS-PAGE of 200 ng of the indicated purified proteins; GST-Rap1p alone or in the presence of a 5-fold molar excess of TBP binding competitors was incubated with GST-TBP-loaded glutathione beads (300 ng of GST-TBP/reaction) as indicated. Bead-bound GST-Rap1p (−−), GST-TBP (---), and bound proteins (black circles) are indicated.
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Given the fact that Rap1p specifically binds to and inactivates TBP, we reasoned that introduction of both Rap1p and TBP into cells carrying the AIT reporter plasmid should diminish the observed TBP-driven reporter gene transcription. We tested this hypothesis by co-overexpressing forms of TBP (WT, R98E, T124R, V161R, R196E, N69S, and L205K) and various isoforms of Rap1p (ΔTox, DBD, and ΔDBD) in cells carrying pADH1-lacZ; all of these proteins were equally expressed (Fig. 6B, right). As seen in the data presented in Fig. 6A, overexpressed TBP increased transcription of the ADH1 promoter-driven lacZ reporter, and two TBP mutants (V161R and N69S) were particularly active in this regard (Fig. 6B, right, white bars). Importantly, co-overexpression of Rap1p DBD (black bars), but not Rap1ΔDBD (light gray bars), significantly reduced TBP-driven AIT in all instances except in the case of the two TBP variants, R98E and R196E, that we had found to be unable to

vivo, and that Rap1p bound TBP through the Rap1p DBD. These results were consistent with our in vitro analyses.

Rap1p DBD Modulates mRNA Gene Transcription in Vivo—

We used two approaches to test whether Rap1p regulated TBP transcriptional function. First, we took advantage of the fact that overexpression of TBP can increase activator-independent transcription (AIT) of certain mRNA-encoding genes, although the exact mechanism responsible for this phenomenon is at present not fully understood. We observed that different TBP mutant proteins variably stimulated AIT (Fig. 6A, top) when expressed in cells carrying either of two reporter plasmids, pADH1-lacZ or pCYC1-lacZ. These results are consistent with the observations of others (69, 72–74) who have shown that certain TBP variants, when overexpressed, stimulate AIT. Immunoblotting showed that all of the TBP proteins tested were equally expressed (Fig. 6A, bottom).

FIGURE 4. Mapping TBP residues that interact with the Rap1p DBD. A, top, ribbon schematic of yeast TBP (96). The single mutant TBP variants tested for Rap1p binding by GST-TBP pulldown (as in Fig. 3C) are labeled. The two TBP residues implicated in Rap1p binding are colored in red. Bottom, linear schematic of TBP truncations used for complementary GST-TBP/Rap1p pulldown experiments. B, purified TBP mutant proteins (lanes 2–19; labeled WT, mutant) were analyzed by SDS-PAGE and visualized by Sypro Ruby staining (top) or subjected to immunoblotting without (middle) or with (bottom) formaldehyde cross-linked samples, X-linked, bottom; asterisks indicate the cross-linked TBP dimers. C, TBP variants were tested for binding to Ad2 MLP DNA by DNase I footprinting, either alone (lanes 1–21), or in the presence of Rap1p (lanes 22–44), TFIIA (lanes 45–67), or TFIIB + Rap1p (lanes 68–92). GST fusions of the TBP variants were used to test Rap1p binding by incubating ~300 ng of GST-TBP mutants bound to glutathione beads in the absence or presence of 1000 ng of Rap1p. Bead-bound proteins were analyzed by Sypro Ruby staining of SDS-PAGE-fractionated samples. GST and GST-TBP fusions are indicated by arrowheads; TBP-bound Rap1p is shown by the arrow labeled Rap1p.
bind Rap1p (cf. Fig. 4). The DBD-containing Rap1ΔTox protein, which can be overexpressed as it lacks the Tox domain, also inhibited TBP-driven AIT, although to a lesser extent than the Rap1p DBD (dark gray bars); the R98E and R196E mutants were also refractory to Rap1p DBD-mediated modulation of AIT. In summary, this data indicated that the Rap1p DBD could modulate the transcriptional activity of TBP in vivo.

Second, to assess whether the Rap1p DBD also affected the transcription of single-copy, endogenous, chromosomal mRNA-encoding genes, we utilized a Cu²⁺-modulated expression shutoff system (Copper-degron) (63) to assess the effects of Rap1p depletion on transcription. A control strain (ZMY60) and Cu²⁺-deletable 

Rap1p target genes (PGK1, ADH1, ADH3, RPS2, RPS3, and RPL3). This effect was RNAP II-restricted because neither 

RDNI gene transcription by RNAPI (supplemental Fig. S2), nor SCR1 and tL(CAA)n (tRNA₆⁰⁰-cua) gene transcription by RNAPIII was affected by Rap1p depletion (Fig. 7A). This experiment was then repeated, but upon endogenous Rap1p depletion various forms of Rap1p (WT, ΔTox, DBD, and ΔDBD) were expressed from the Rap1p enhancer/promoter at physiological levels (supplemental Fig. S1D). RNA was again prepared and analyzed for transcript levels. We found that any form of co-expressed Rap1p that carried the DBD (i.e. WT, ΔTox, and DBD) blocked the Cu²⁺-induced up-regulation of mRNA gene transcription (Fig. 7B), presumably by sequestering TBP. Furthermore, and importantly, the extent of Rap1p-DBD-mediated modulation of mRNA gene transcription was proportional to the levels of these Rap1p variants (supplemental Fig. S2B). Collectively this series of four experiments argued that in vivo, Rap1p, working through DBD-mediated interactions with TBP, modulated mRNA gene transcription.

Up-regulation of mRNA Levels Seen in LEV391 Cells upon Rap1p Depletion Is Associated with Increased Recruitment of the Transcription Machinery—To demonstrate that the observed increases in mRNA levels seen upon Rap1p depletion were also refractory to Rap1p DBD-mediated inhibition of AIT. 

**FIGURE 5. TBP and Rap1p are complexed in vivo.** A, left, WCE were prepared from yeast strains expressing control (cognate WT, RAP1) or various tagged forms of Rap1p from the chromosomal RAP1 locus (C-terminal TAP tag, RAP1-TAP; N-terminal TAP tag, TAP-RAP1; N-terminal Myc tag, Myc-RAP1; N-terminal HA tag, HA-RAP1). Equivalent amounts of WCE proteins were fractionated by SDS-PAGE, and either stained directly (Sypro Ruby WCE) or electrotransferred to membranes and probed with anti-Rap1p IgG, anti-TAP IgG, anti-Myc IgG, anti-HA IgG, or anti-TBP IgG; full-length Rap1p variants are indicated by dark gray bars the Rap1p DBD (bottom); the R98E and R196E mutants also inhibited TBP-driven AIT, although to a lesser extent than 

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**Figure 6.** Rap1p DBD modulates mRNA reporter gene transcription in vivo. A, yeast strain W303a, containing one of two plasmid-born lacZ reporter genes (pADH1-lacZ, pCYC1-lacZ), was transformed with empty vector (–) or vector expressing the indicated HA-tagged forms of TBP. Top, -fold activation of β-galactosidase induction in cells overexpressing either no TBP, or the indicated forms of TBP; expression was from pADH1-lacZ (light gray) or pCYC1-lacZ (defined as 1.0; dark gray bars) reporters. Data from three simultaneous independent experiments is shown along with S.E. Bottom, expression of TBP variants as measured by immunoblotting WCEs with anti-HA monoclonal antibody. B, yeast strain W303a, carrying a URA3-marked pADH1-lacZ reporter was transformed with a HIS3-marked plasmid, which was either empty (–) or expressing HA-tagged WT, R98E, T124R, V161R, R196E, N69S, or L205K mutants of TBP, and/or a LEU2-marked plasmid, which was either empty (–) or expressing FLAG-tagged ΔTox (dark gray bars), DBD (black bars), or ΔDBD (light gray bars) Rap1p variants. β-Galactosidase activity was measured and plotted as -fold activation by TBP overexpression relative to the amount of β-galactosidase expressed in cells carrying the empty vectors. The effect of Rap1p variants upon TBP-induced AIF was calculated as above; right, immunoblot showing protein expression levels of the Rap1p and TBP variants.

We observed that overexpression of the Rap1p DBD, but not the Rap1p-ΔDBD variant, dominantly inhibited yeast cell growth (Fig. 8B).

**Discussion**

The evolutionarily conserved TATA-binding protein is essential for most nuclear gene transcription in eukaryotes (75, 76). By binding to promoter DNA near the start site of transcription TBP catalyzes PIC formation on mRNA-encoding genes by facilitating the cooperative interaction of RNAPII and the GTFs with the promoter. TBP may be escorted to promoters in the form of the TFIID coregulator, or via other coregulators (i.e. Mediator, SAGA, and Mot1p). On RNA polymerase I- and III-transcribed genes the GTFs SL1, SNAPC, and TFIIIB operate in a similar fashion by chaperoning TBP to target promoters for subsequent PIC assembly and transcription.

Consistent with the central role of TBP in transcription, its activity is subject to exquisite control by a plethora of TBP-interacting proteins. These include NC2, TFIIA, TFIIIB (77), Mbf1p (78), Sptp3p, Spt8p, and Ada1p subunits of SAGA (79, 80), TFIID Taflp and TAND (58, 59, 81, 82), Med8p and Med20p subunits of Mediator (83), Bf1p subunit of TFIIIB (84), TAF48 subunit of SL1 (85), N-terminal domain of Mot1p (86), SNAP190 subunit of SNAPC (87), and the NOT complex (88). Interestingly, many of these TBP-targeted factors both activate and repress transcription (76).

Intriguingly, many of these disparate TBP-directed regulators is the fact that essentially all utilize direct protein–TBP interactions that target amino acid residues in and around the concave DBD of TBP to regulate TBP activity. Here we report our discovery of a new member of this family of TBP-targeted transcriptional regulatory proteins, Rap1p.

Addition of Rap1p to TBP-TATA DNA binding reactions inhibited the binding of free TBP, but not TBP-TATA complexes, whereas TFIIA, but not TFIIIB, protected TBP from inhibition by Rap1p; TBP-Rap1p interaction was bimolecular (Fig. 1). The basic Rap1p DBD (pl 8.6) bound directly to the basic TBP (pl 10.2) to inhibit DNA binding (Fig. 2) and the TFIIA-, TATA-, TAND-, and NC2-interaction domains of TBP were involved in binding (Fig. 3). Mapping indicated that two residues, Arg-98 and Arg-196, on either side of the concave DBD of TBP specifically interacted with the Rap1p DBD providing a molecular explanation for how Rap1p–TBP complex formation could block TBP-DNA binding (Fig. 4). Not only did TBP and Rap1p interact specifically and efficiently in vitro, we demonstrated by co-IP that under normal expression levels TBP could be transcribed to Rap1p in vivo. Both untagged endogenous TBP and ectopically co-expressed tagged-TBP interacted with Rap1p in vivo (Fig. 5). Although these co-IP experiments did not indicate if this interaction was direct, we did
show that like the in vitro situation, TBP-Rap1p co-IP depended upon the Rap1p DBD. Furthermore, given our data showing that the Taf1p TAND blocked interaction between TBP and Rap1p (Fig. 3), and, that previously we have shown that TFIIID directly binds both Rap1p DBD and C-terminal domains with roughly equal affinity, leads us to believe that TBP-Rap1p co-precipitation does not involve TFIIID (cf. Ref. 55). Most importantly, we showed that the Rap1p DBD functionally interacted with TBP in vivo by demonstrating that forms of Rap1p carrying the DBD specifically regulated both TBP-driven activation (Fig. 6) as well as endogenous chromosomal mRNA gene transcription (Figs. 7 and 8). Not surprisingly then, overexpression of the DBD, devoid of any Tox domain sequences, dominantly inhibited yeast cell growth (Fig. 8).

Collectively, our data lead us to entertain a model wherein we hypothesize that Rap1p can act as a "TBP buffer," serving to sequester TBP as an inactive TBP-Rap1p complex under conditions of excess free TBP. Such complex formation could have multiple consequences (see below). It should be noted that like TBP, Rap1p is an abundant protein (~2 × 10^4 molecules/cell). However, the critical question for both proteins, indeed for most interacting nuclear transcription proteins is: what fraction of either TBP or Rap1p is free? It is clear from our data that...
Rap1p Regulation of TBP Activity

TBP-Rap1p complex formation excludes functional TBP interactions such as TATA DNA binding and holo-TFIID complex formation; this is schematically illustrated in Fig. 9A.

One consequence of Rap1p-TBP complex formation could be that Rap1p operates mechanistically in a fashion similar to other TBP-directed transcriptional regulators such as NC2, Mot1p, and SAGA coregulators, that all form stable complexes with TBP (77, 79, 89). A corollary of this model is that by associating or dissociating from TBP, Rap1p will negatively or positively affect transcription by decreasing or increasing the pool of free TBP. In vivo, TBP can clearly shuttle from TFIIIB to TFIID, but apparently fails to shuttle efficiently from TFIID to TFIIIB, or from TFIIIB to the yeast SL1-like RNAP I GTF (90). Moreover, we have previously shown that TBP dynamically associates with TFIID in vitro (5). These data are all consistent with our observations presented in Figs. 7, 8, and supplemental S1 and S2, which demonstrated an RNAP II-specific effect of Rap1p depletion on nuclear gene transcription. In this case, when Rap1p levels dropped, the amount of TBP available to interact with the 14-subunit TFIID TAF complex could go up, presumably increasing the amount of functional, transcriptionally competent holo-TFIID, leading to stimulation of mRNA gene transcription (cf. Fig. 9B). Similar arguments can be made for TFIID-independent transcription, for example, genes driven by the SAGA-TBP, Mot1p-TBP, or other coregulator-TBP complexes. Finally, one cannot rule out the fact that such non-TFIID coregulator-TBP complexes may (spuriously) drive mRNA gene transcription under the artificial TBP excess conditions we have generated here using the LEV391 Rap1p-shut off strain. Dramatic changes in essential coregulator and/or activator requirements have been seen before in systems genetically manipulated to deplete specific transcription proteins (91–93). Thus, it is possible that under physiological conditions Rap1p normally functions to chaperone TBP transfer between TFIIIB and TFIID, thereby fine-tuning mRNA, tRNA, and 5 S rRNA gene transcription. Rap1p has been implicated in the regulation of the most actively transcribed genes in yeast cells, the RP- and glycolytic enzyme-encoding genes (18), hence Rap1p and Rap1p-driven mRNA gene transcription is subjected to growth rate-sensitive signaling pathways (16) and thus potentially ideally situated to modulate transcription via interaction with TBP.

A second possible consequence of Rap1p-TBP complex formation is that this interaction could antagonize the negative action(s) of any or all of the other known TBP-targeted transcriptional regulators. Indeed, we have shown here (cf. Fig. 3) that TFIIA, TATA DNA, Taf1p TAND, and NC2 all potentially prevented the binding of Rap1p to TBP. Hence, one function of Rap1p-TBP complex formation may be to affect the interplay of these TBP-targeted regulators with TBP under appropriate conditions. Any molecule that dramatically alters intranuclear distribution of TBP between specific, TBP-linked TBP-TAF complexes will effect global nuclear gene expression (94). Experiments are in progress to directly test the ability of Rap1p to influence the interactions between TBP and Mediator, SAGA, Mot1p, NC2, and other TBP-binding proteins.

Finally, third, Rap1p-TBP complex formation could control active/free TBP levels by affecting TBP dimerization. TBP dimerization has been hypothesized to importantly contribute to the control of mRNA gene transcription in vivo, and one of the molecules that blocks TBP-Rap1p complex formation, TFIIA, has been implicated in facilitating TBP-TBP dimer dissociation (95). If TBP dimerization contributes to regulation of free TBP levels, then Rap1p may help pull dimeric TBP, via mass action, to the monomer, the form that is competent to interact with TAFs to form functional GTFs and/or other functional TBP-containing coregulators. If this is the case, our model would need to include one additional TBP state, TBP-TBP dimers, in equilibrium with the Rap1p-TBP complex and monomeric (i.e. functional, non-dimerized) TBP. Our future work will dissect the molecular mechanisms by which the novel TBP regulator, Rap1p, serves to modulate TBP function within

FIGURE 9. TBP-Rap1p interactions. A, binding interactions of TBP to TATA DNA, Taf1p TAND, and Rap1p are mutually exclusive. Shown are three TBP binary interactions: I, TBP-TATA DNA; II, TBP-TFIIID Taf1p TAND; and III, TBP-Rap1p. All three high affinity interactions involve the conserved DNA binding surface of TBP, hence binding of TBP to any one of these ligands blocks binding by the others (cf. Fig. 3). Listed are the Kd values and complex half-lives we have measured for complexes I and II (58, 59, 65, 68, 69); complex III, N.D., not yet determined. B, model illustrating the linkage between the Rap1p-TBP complex and TFIID-driven mRNA gene transcription (1). If the in vivo levels of Rap1p are decreased (X), either naturally or by genetic depletion (as with the copper degron in strain LEV391; Fig. 7 and supplemental Fig. S2) there will be a corresponding increase in free TBP (2); this drop in Rap1p causes an increase in the intranuclear [TBP] (3). Because TBP dynamically interacts with the 14-subunit TAF complex (65), an increase in the concentration of TBP could, by mass action (4), increase the formation and concentration of holo-TFIID (TFIID). This increase in holo-TFIID could then drive mRNA gene transcription (TnX (5). Note that an alternative to increased holo-TFIID (or in addition to), increased concentrations of TBP could also stimulate formation of SAGA-TBP, mediator-TBP, Mot1p-TBP etc., complexes that themselves could also drive an increase in mRNA gene transcription in a physiological or non-physiological fashion.

TBP-Rap1p interactions

I. TBP-TATA DNA

II. TBP-TFIIID Taf1p TAND

III. TBP-Rap1p

Kd = 5nM

t1/2 = 15-30 min

Kd = 1nM

t1/2 = 15 min

Kd = N.D.

t1/2 = N.D.
the nucleus, with a particular eye toward placing Rap1p within the broader context of other TBP-targeted transcriptional coregulators.

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REFERENCES

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