High Affinity Interaction of Yeast Transcriptional Regulator, Mot1, with TATA Box-binding Protein (TBP)*

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Yeast Mot1, an essential ATP-dependent regulator of basal transcription, removes TATA box-binding protein (TBP) from TATA sites in vitro. Complexes of Mot1 and Spt15 (yeast TBP), radiolabeled in vitro, were immuno-precipitated with anti-TBP (or anti-Mot1) antibodies in the absence of DNA, showing Mot1 binds TBP in solution. Mot1 N-terminal deletions (residues 25–801) abolished TBP binding, whereas C-terminal ATPase domain deletions (residues 802–1867) did not. Complex formation was prevented above 200 mM salt, consistent with electrostatic interaction. Correspondingly, TBP variants lacking solvent-exposed positive charge did not bind Mot1, whereas a mutant lacking positive charge within the DNA-binding groove bound Mot1. ATPase-defective mutant, Mot1(D1408N), which inhibits growth when overexpressed (but is suppressed by co-overexpression of TBP), bound TBP normally in vitro, suggesting it forms nonrecyclable complexes. N-terminal deletions of Mot1(D1408N) were not growth-inhibitory. C-terminal deletions were toxic when overexpressed, and toxicity was ameliorated by TBP co-overproduction. Residues 1–800 of Mot1 are therefore necessary and sufficient for TBP binding. The N terminus of 89B, a tissue-specific Drosophila Mot1 homolog, bound the TBP-like factor, dTRF1. Native Mot1 and derivatives deleterious to growth localized in the nucleus, whereas nontoxic derivatives localized to the cytosol, suggesting TBP binding and nuclear transport of Mot1 are coupled.

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In yeast (Saccharomyces cerevisiae), as in other eukaryotes, many positive and negative regulatory factors act in concert to ensure that transcription initiation by RNA polymerase II (pol II) is controlled correctly (1). Promoter accessibility is dictated by chromatin structure, which is modulated by chromatin-remodeling complexes and histone-modifying enzymes (2). Once a promoter is available, formation of a preinitiation complex begins with binding of TFIIID, a complex composed of the TATA box-binding protein (TBP) and several TBP-associated factors (TAFs) (3). TFIIID binds to the TATA box sequence present at nearly all yeast promoters (4, 5). Formation of a TFIIID-DNA complex creates a platform for assembly of the remaining general transcription factors and pol II (6–8).

Transcriptional activators stimulate transcription initiation via recruitment of the pol II preinitiation complex to a promoter either through direct interaction with TBP, TFIIIB, and/or components of the pol II holoenzyme (9, 10) or via intervening “mediator” complexes (11). Considerable evidence highlights the importance of activator recruitment of TBP. For example, by using a TBP derivative with altered DNA binding specificity, it was shown that activator-dependent engagement of TBP at a promoter is rate-limiting for transcription in vivo (12). Also, fusion of TBP to the DNA-binding domains of either LexA or Gal4 allows high level transcription from LexA- or Gal4-binding sites in the absence of any other activator (13–15). Finally, as shown by in vivo cross-linking techniques, promoter occupancy by TBP correlates with transcriptional activity (16, 17).

Because promoter occupancy by TBP is central to gene activation (18), TBP is also a prime target of transcriptional inhibitors. A conserved heterodimeric repressor, NC2/Dr1/DRAP, binds TBP via a histone-like fold, preventing TBP association with TFIIA and/or TFIIIB, thereby repressing transcription (19–22). S. cerevisiae cells lacking NC2α (BUB6/NCB1 gene product) grow very poorly; cells lacking NC2β (NCB2 gene product) are inviable (21, 23).

Another class of negative transcriptional regulator targeting TBP is the yeast mot1-1 gene product. Like NC2, Mot1 is essential for viability and evolutionarily conserved. Mot1 homologs exist in Drosophila (55) and humans (63, 64). A recessive temperature-sensitive mutation (mot1-1) elevated transcription of a plasmid-borne reporter gene in the absence of its

* The abbreviations used are: pol II, eukaryotic RNA polymerase II; AMP-P-NH-P, 5-adenylyl-imidodiphosphate; BSA, bovine serum albumin; dTBP, D. melanogaster TATA box-binding protein; dTRF1, D. melanogaster TBP-related factor-1; DTT, dithiothreitol; GST, Schistosoma japonicum glutathione S-transferase; HA, influenza virus hemaglutinin; mAb, monoclonal antibody; Myc, product of the c-myc protooncogene; NLS, nuclear localization sequence; ORF, open reading frame; Raf, raffinose; Suc, sucrose; TAF, TBP-associated factor; TBP, TATA box-binding protein; UAS, upstream activating sequence; and, yTBP, Saccharomyces cerevisiae TATA box-binding protein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair.

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normal activator and also increased transcription from several chromosomal pol II-dependent genes, as well as from a basal promoter lacking any upstream activation sequence (UAS) (24).

An independently isolated allele (mot1-1033) had a similar effect on another pol II gene (25). Another recessive allele (bur-3,1) for “bypass UAS requirement,” now mot1-301) was isolated by selecting for mutations permitting transcription from the SUC2 gene promoter lacking its UAS (23, 26).

Consistent with a primary role as a negative regulator, Mot1 was found to be the factor responsible for an ATP-dependent inhibitory activity (ADI) in nuclear extracts (27) that removes TATA box-bound TBP in an ATP-dependent manner (28). Although TBP stimulates RNA synthesis by all three RNA polymerase types in transcription reactions in vitro (29), the presence of Mot1 decreases transcription of pol II genes, but not pol I or pol III genes (28). Likewise, Mot1 is required for repression in vitro of LEU2 expression by the negative regulator, Leu3 (30). Mot1 was also identified in nuclear fractionation studies as a component (Taf170) of TBP-containing complexes distinct from TFIID (31).

The C-terminal domain of Mot1 possesses the seven signature motifs of a superfamily of helicases and nucleic acid-dependent ATPases (32), many of which modulate the state of assembly of protein-nucleic acid complexes (33). Mot1 is the prototype of a distinct class that includes Snf2 (yeast), ERCC6/CSB (human), Brahma (Drosophila), HepA (Escherichia coli), and others (24, 34). Despite this homology, no helicase activity has been reported for any member of the Mot1/Snf2 group. Mot1, purified to apparent homogeneity, does not exhibit even local DNA strand-unwinding activity but does retain full ability to release TBP bound to a TATA box in an ATP-dependent manner (35). Prior studies (36) suggested that contacts with DNA do not play a critical role in Mot1-TBP interaction. Here we have explored this issue in detail and present experiments performed both in vivo and in vitro that define the requirements for, and quantify the affinity of, the physical association between Mot1 and TBP. We also determined the subcellular localization of Mot1, and the role of TBP interaction in Mot1 compartmentation.

### EXPERIMENTAL PROCEDURES

**Growth Conditions, Strains, and Recombinant DNA Method**—Strain W303-1A (MATa ura3-1 his3-11,15 ade2-1 leu2-3,112 trp1-1 can1-100) was used for all experiments, unless otherwise indicated. For immuno-fluorescence, an otherwise isogenic MATa/MATa diploid (W303D) was used. Strain KHY18 was constructed in the following manner. Strain YPH501 (37) was transformed (38) with an XhoI-Not1 fragment of plasmid pKH4 containing the mot1-12::LEU2 allele in which the LEU2 gene replaces codons 79–1822 of the MOT1-coding sequence (39). After confirming correct transplacement of the MOT1 locus on one homolog of chromosome XVI by Southern blotting, the resulting LEU2+ diploid (KHY1) was transformed with pRSMot1, a derivative of the URA3-marked CEN-based vector, pRS316 (37), containing a 7.1-kb genomic BglII fragment expressing MOT1 from its endogenous promoter (24). After sporulation and tetrad dissection, one of the resulting URA3::LEU2::MATa spore clones was designated KHY18 and was used to study this two-site-directed mutants (mot1-462 and mot1-503) generated in this study (see below). Strain WPY1 was generated by transformation of strain W303D with a BamHI-SmaI fragment of plasmid pKA23 containing the spt15Δ::LEU2 mutation, in which a portion of the SPT15 coding sequence has been replaced with the LEU2 gene (40). After selection of LEU2 transformants and verification for proper integration at the SPT15 locus using the polymerase chain reaction (PCR) with appropriate primers, WPY1 was transformed with a TRP1-marking CEN-based plasmid expressing wild-type SPT15 from its endogenous promoter (pUN4511) or the same plasmid expressing either of two TBP mutants (K133L,K138L or K133L,K145L) (41). After sporulation, the cells were treated with ethyl ether to enrich for spores (42), and LEU2+ TRP1+ haploids carrying the spt15Δ::LEU2 mutation, maintained by low copy plasmids expressing TBP or TBP mutants, were selected on medium lacking Leu and Trp.

Yeast cells without plasmids were grown at 30 °C in rich medium (YP), and cells with plasmids were grown in minimal medium (SC) lacking the appropriate nutrient(s) to maintain selection, as described (43), using glucose (Glc) as the carbon source unless otherwise indicated. Solid media contained either 2% Glc or 2% galactose and 0.2% sucrose (Gal/Suc). For expression of genes from the galactose-inducible GAL1 promoter in liquid media, cells were pregrown in SC containing 2% rafinose (Raf) as the carbon source, and then galactose (Gal) was added to 2% to initiate induction. Plasmids were introduced into yeast cells using a modification of the lithium acetate transformation procedure that utilizes single-stranded carrier DNA (38). Recombinant DNA manipulations were carried out using standard techniques (44).

### Construction of Plasmids for Expression in Yeast—To facilitate immuno-detection of Mot1, DNA encoding the 16-residue c-Myc epitope (LEEPQKLISEEDLRLKR) recognized by the monoclonal antibody (mAb) 9E10 (45) was fused in-frame to the 3’-end of the MOT1 coding sequence, using a PCR-based method that exploits 3 primers (46) as follows: an upstream primer (JDM26), a MOT1/Myc joiner primer (JDM25), and a Myc epitope-containing primer (MYC) (Table I). The resulting 674-base pair PCR product was introduced into the SmaI site of plasmid pRS316 (37) by blunt-end ligation and verified by DNA sequencing. The resulting product was digested with BstXI and introduced into BesI-digested pRSMot1, yielding pRSMot1Myc. Addition of the Myc tag did not detectably alter Mot1 function since pRSMot1 fully complements a mot1Δ mutation (data not shown). A Myc-tagged version of the dominant-negative ATPase-defective MOT1(D1408N) allele (28) was prepared in the same fashion, yielding pRSMot1- (D1408N)Myc.

### Mot1-TBP Interaction

| Name          | Sequence (5’ to 3’)                                      |
|---------------|--------------------------------------------------------|
| JDM6          | AACCTCCTAGAAAGATGATG                                  |
| JDM25         | AAGTAGGTTTCGAAAGCTCGTCGCTG                            |
| JDM26         | GACATGGTTGAAATG                                       |
| KHP5          | GGCTGCTGATCAGGGCCAGTGCAACTAA                         |
| KHP6          | GGGCCGGTTGAGCTGTTGAGTTAGGATGAAATG                    |
| KHP9          | GGGCCGGTTGAGCTGTTGAGTTAGGATGAAATG                    |
| KHP12         | AATTAAACCTCATGAAAGGGAGCATTGTCCTTTAAAGGAAGTCAATAC     |
| KHP13         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| KHP14         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| KHP15         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| KHP16         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| KHP22         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| KHP23         | AATTTAACCCTCATTAAAAAGGAGCATTGTCCTTTAAAGGAAGTCAATAC   |
| MYC          | TTAGGGACGGCTACTGAAAGGAGACGGCTACTGAAAGGAGACGGCTACTGAA |
| T7           | TTCGGGAGCTACTGAAAGGAGACGGCTACTGAAAGGAGACGGCTACTGAA   |
| T7           | GATTAACGACTCACTAGAGCCGAGCCGACTCACTAGAGCCGACTCACTAGAGC |

| Name          | Sequence (5’ to 3’)                                      |
|---------------|--------------------------------------------------------|
| MYC          | TTAGGGACGGCTACTGAAAGGAGACGGCTACTGAAAGGAGACGGCTACTGAA |
| T7           | GATTAACGACTCACTAGAGCCGAGCCGACTCACTAGAGCCGACTCACTAGAGC |
Mot1-TBP Interaction

With XhoI and SalI and relocating the vector. The PCR product generated using primers KPH9 and JDM6 with pKH2 as the template was digested with XhoI and SalI and introduced into pKH2 digested with the same enzymes to create plasmid pKH29, which expresses mot1Δ1-1620 from the T3 RNA polymerase promoter.

Immunoprecipitation—Proteins to be used in co-immunoprecipitation experiments were produced by coupled in vitro transcription and translation in the presence of [35S]Met (PerkinElmer Life Sciences) using the PromoGen TNT™ Coupled Reticulocyte Lysate System, according to the manufacturer’s directions. To increase yield, 0.2 mg/ml yeast RNA (Sigma) was added to each translation mixture. Purified or PCR-derived linearized DNA templates were transcribed with phenol-chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. After translation, proteins were partially purified by precipitation with 50% ammonium sulfate (50), which also allowed for quantification of translation yield as described in detail elsewhere (51).

Briefly, the concentration of translated protein can be estimated by the percent incorporation of [35S]Met, the number of Met residues known to be present in the sequence of the protein, the molecular weight of the protein, and the concentration of unlabeled methionine in the extract (~5–10 μM). The ammonium sulfate precipitates were resuspended in Buffer A (20 mM Tris-HCl (pH 7.5), 75 mM potassium acetate, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Tween 20, and 12.5% glycerol) at a volume equal to the starting volume. The solution was then incubated at 4 °C for 1 h on a roller drum, the agarose beads were collected by centrifugation for 5 min in a microcentrifuge. The supernatant solution was removed by aspiration using a 25-gauge needle, and the pellets were washed three times with 1 ml of ice-cold Buffer A. The bead-bound immune complexes were resuspended in SDS sample buffer, boiled, and resolved on a 10% SDS-polyacrylamide gel, along with a lane containing [35S]labeled molecular weight markers (Amersham Pharmacia Biotech). After electrophoresis, the gel was dried and exposed to X-ray film for 2–5 days. The film was scanned and analyzed using an imaging system (ECL™, Amersham Pharmacia Biotech) as recommended by the manufacturer.

Immunoblotting— Cultures of strain W303-1A harboring plasmids containing the various MOT1Myc derivatives were grown to an approximate density of 8 × 10⁶ cells/ml in SC medium lacking uracil, induced by addition of 0.5% Gal, and grown for an additional hour to be harvested by centrifugation and washed once with ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml pepstatin A, 1 mM DTT). All subsequent steps were carried out at 4 °C. An amount of cells (equivalent to 10–15 mg of total protein) was resuspended in 200 μl of lysis buffer and lysed by vortex mixing with glass beads as described in detail (53). Unbroken cells and large cell debris were removed by low speed centrifugation (2000 rpm for 5 min in a Sorvall SS-34). For immunodetection of Mot1, a sample of the lysate equivalent to 30 μg of total protein, as determined by the dye-banding method of Bradford (54), was resolved by SDSPAGE on an 8% gel, transferred to a nitrocellulose filter, and incubated with anti-c-Myc mAb 9E10 at a 1:300 dilution. Secondary antibody (anti-c-Myc mAb 9E10) was used at a 1:300 dilution. Secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse Fab’₂ fragment; Jackson Immunoresearch) was used at a 1:100 dilution.

Bacterial Expression and Use of GST Fusions for Protein Binding

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Mot1-TBP Interaction

Fig. 1. Mot1 and Mot1(D1408N) coimmunoprecipitate with yTBP. [35S]Met-labeled Mot1, Mot1(D1408N), and Rad3 (as a control) were produced by in vitro transcription and translation and partially purified by ammonium sulfate precipitation. For each coimmunoprecipitation reaction, 0.5 pmol of the indicated protein was incubated in either the absence (lanes 4, 6, and 8) or the presence (lanes 5, 7, and 9) of a 2-fold molar excess of purified unlabelled yTBP at 4°C for 30 min and then with 2 μl of rabbit polyclonal α-yTBP antibodies for 1 h at 4°C. The resulting immune complexes were recovered by binding to protein A-agarose beads. After brief centrifugation, the supernatant solution was removed, and the beads were washed once with buffer. The bead-bound immune complexes were solubilized by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography, along with portions (2%) of the initial amount of material that was added to each reaction (Input, lanes 1-3).

RESULTS

Direct and High Affinity Interaction of Mot1 and yTBP—To determine whether Mot1 and yTBP associate in the absence of DNA, radiolabeled Mot1 (and various derivatives) was produced by coupled in vitro transcription and translation and mixed in solution with a 2-fold excess of purified bacterially expressed yTBP. The mixture then was precipitated with bead-bound anti-yTBP antibodies, and the amount of coimmunoprecipitated Mot1 was examined (Fig. 1). As one control, Mot1 alone (in the absence of yTBP) was treated in the same way to measure nonspecific binding to the beads. The amount of antiyTBP antibodies used was sufficient to capture 20–30% of the input, lanes 1–3.

The calculated molecular mass of Mot1 is 210 kDa; however, the full-length protein prepared by in vitro translation (28) or the native protein purified from yeast (35, 36) migrates on SDS-PAGE with an apparent size of ~180 kDa, compared with standard molecular weight markers. Hence, as expected, radiolabeled Mot1 and an ATPase-defective point mutant, Mot1(D1408N) (28, 39), appeared as a prominent 180-kDa doublet corresponding to full-length and near full-length protein (with minor amounts of lower Mr, species representing both...
Conversely, addition of an excess of a TATA-containing DNA fragment did not increase the amount of bead-bound Mot1 in either the absence or presence of yTBP (data not shown). Finally, as a negative control, radiolabeled Rad3, a demonstrated 90-kDa helicase (in the same superfamily, but only distantly related to Mot1) and a known component of yTFIIH (48), showed only nonspecific adsorption to the beads that was unaffected by yTBP (Fig. 1, lanes 8 and 9). By using the same method, rabbit polyclonal antibodies directed against the C-terminal domain of Mot1 (28, 39) were able to coimmunoprecipitate yTBP in either the absence or presence of ethidium bromide (data not shown). These results clearly demonstrated first, formation of Mot1-yTBP complexes in dilute solution, and second, that this interaction does not require mutual binding of these proteins to DNA.

An approximate $K_{d}$ for the Mot1-yTBP-binding reaction could be calculated by determining the concentrations of Mot1 and yTBP present in the reaction from their specific radioactivity, by estimating the fraction of the total input yTBP immunoprecipitated, and by quantitating the fraction of the total input Mot1 coimmunoprecipitated (see Ref. 51 for additional details of this method). The $K_{d}$ value, estimated from many such experiments conducted at different input levels of the reactants, always fell in the range 20–50 nM. This value may underestimate Mot1-yTBP binding affinity because some antibodies in the polyclonal anti-yTBP antiserum could interfere with complex formation and because Mot1 concentration in each reaction was most likely overestimated (given that a significant portion of the radioactive species was not full-length and was unable to interact with TBP; see Fig. 1). Nonetheless, the estimated $K_{d}$ is reasonably good agreement with a value ($K_{d}$ = 100 nM) determined from a totally independent method, the concentration dependence of the stimulation of the ATPase activity of purified Mot1 by purified yTBP (35).

Radiolabeled Mot1(D1408N) (28, 39) displayed the same binding behavior as wild-type Mot1 (Fig. 1, lane 6 versus lane 7), indicating that catalytic activity of the C-terminal ATPase domain is not required for yTBP binding. Consistent with this result, addition of ATP or a nonhydrolyzable ATP analog (AMP-P-NH-P) neither enhanced nor reduced binding of either normal Mot1 or Mot1(D1408N) to yTBP in this assay (data not shown). Mutation of the equivalent residue in another member of this ATPase superfamily (eIF-4A) prevents ATP hydrolysis but not ATP binding (59).

Mapping the Mot1-binding Region of yTBP—In the absence of ATP, purified Mot1 forms a ternary complex with yTBP bound to TATA-containing DNA; in the presence of ATP, Mot1 acts catalytically to dissociate yTBP-DNA complexes (35, 36). The Mot1-containing ADI fraction of yeast nuclear extracts was unable to dissociate yTBP-DNA complexes prepared using yTBP variants with point mutations in the so-called basic region (27), which corresponds to a solvent-exposed a-helix (Helix 2) in the C-terminal lobe of yTBP (52). Mutations in this same region of yTBP decrease its ability to form complexes with TFIIA (41), and TFIIA competes with Mot1 for binding to DNA.
yTBP-DNA complexes (28). To determine whether mutations in Helix 2 affect Mot1-yTBP interaction in solution and in the absence of DNA, three yTBP variants carrying mutations in this segment (Fig. 2A), Spt15(K133L,K138L), Spt15(K133L,K145L) (41), and Spt15(K138T,Y139A) (49), and a mutant, Spt15(R105H), that alters a residue on the surface of yTBP that contacts TATA DNA (52), were tested for their ability to bind Mot1 by the coimmunoprecipitation method.

Radiolabeled forms of wild-type yTBP and all four mutants were produced in equivalent amounts (Fig. 2B, Input) and mixed in 2-fold excess with radiolabeled Mot1. As observed before, only a trace of Mot1 was nonspecifically adsorbed to the anti-yTBP beads, whereas in the presence of wild-type yTBP, the amount of bead-bound Mot1 increased more than 10-fold (Fig. 2B). The mutant, Spt15(R105H), with an alteration within the concave DNA-binding face of yTBP, bound Mot1 as well as normal yTBP. In contrast, all three mutants with alterations in Helix 2 were unable to bind Mot1 (Fig. 2B), as reproducibly observed in three independent trials (Fig. 2C). Thus, contacts in Helix 2 of yTBP mediate its interaction with Mot1.

Mot1-yTBP Interaction Is Largely Electrostatic—The fact that Mot1 binding was abolished by mutations that eliminated positive charge on yTBP suggested that the interaction has an electrostatic component. Competing salt ions weaken electrostatic interactions but strengthen hydrophobic interactions (60). Consequently, radiolabeled Mot1 and radiolabeled yTBP were mixed in the presence of no additional salt (above that present in the buffers used to prepare these proteins) or in the presence of increasing concentrations of potassium acetate, and their interaction was assessed by the coimmunoprecipitation method. As ionic strength increased, the amount of Mot1 bound to yTBP decreased; in contrast, even the highest level of salt tested had no effect whatsoever on the efficiency of immunoprecipitation of yTBP by the anti-yTBP antibodies (Fig. 3A). Strongest interaction of Mot1 with yTBP was observed in the absence of added salt; however, under these conditions, there was slightly more nonspecific interaction of Mot1 with the antibody-coated beads than at the lowest salt concentration (75 mM). Consequently, subsequent immunoprecipitations were routinely performed in 75 mM potassium acetate. The striking monotonic relationship between increasing salt concentration and decreasing Mot1 recovery (Fig. 3B) suggests that Mot1-yTBP binding is mediated mainly by electrostatic contacts.

Genetic Mapping of the yTBP-binding Region of Mot1—The C-terminal domain of Mot1 (residues 1255–1867), when expressed in a MOT1 strain (W303-1A). Equivalent expression was expected because all constructs were generated from the same parental plasmid and, thus, shared the same marker, the same origin of replication, the same promoter (the Gal-inducible and Glc-repressible GALI promoter), the same 5’-untranslated and initial N-terminal sequence (except for Mot1(D11-1100), which is initiated from an internal Met codon), and the same C-terminal tag. Indeed, on Gal-containing medium, all of the internal deletions and C-terminal truncations constructed, whether in Mot1 (Fig. 4A) or Mot1(D1408N) (data not shown), were expressed at similar levels and at their expected molecular masses, as judged by SDS-PAGE and immunoblotting with anti-Myc mAb 9E10 (Fig. 4B).

To examine the effect of overexpression, cells carrying each construct were serially diluted and spotted on either Glc- or Gal-containing agar medium. On the repressing Glc medium, all derivatives grew well and indistinguishably (data not shown). On Gal medium, overexpression of Mot1 had no deleterious effect on growth (Fig. 5A, left panel), compared with cells carrying empty vector, whereas overexpression of Mot1(D1408N) markedly inhibited growth (Fig. 5A, right panel), as observed previously (28, 39). Similarly, two C-terminal truncations that removed the catalytic domain of Mot1 were growth inhibitory when overexpressed (Fig. 5A, left panel, bottom); one, Δ1262–1867, was nearly as toxic as Mot1(D1408N). Likewise, in the context of either Mot1 or Mot1(D1408N), derivatives (Δ1090–1259 and Δ802–1259) that retained the first 800 N-terminal residues of Mot1 displayed a detectably deleterious effect on growth (Fig. 5A, left and right panels), whereas all of the remaining constructs, in which portions or all of these
residues were removed (Δ494–801, Δ244–1280, Δ25–491, Δ25–243, and Δ1–1100), had no effect on growth. Given that all inhibitory derivatives had the same phenotype in the context of either Mot1 or Mot1(D1408N), these deletions presumably abolish Mot1 activity (and presence of the D1408N mutation is unknown). The function of the region between residues 801 and 1254 required for both yTBP binding and for nuclear localization. We have indicated. Results presented here demonstrate that residues 1–801 are required for both yTBP binding and for nuclear localization. We have shown previously that the C-terminal domain is a catalytically active ATPase (28). The function of the region between residues 801 and 1254 is unknown. Bottom, relative positions of the conserved ATPase domain (solid box) and the putative bipartite NLS (black stripe) are indicated. The extent of the sequence removed is indicated by the numbers to the left of each construct and by the missing area (connected by thin lines for internal deletions). All constructs were tagged with a C-terminal c-Myc epitope (not shown). A, wild-type yeast cells (strain W303-1A) were transformed with an empty multicopy vector (YEp352GAL) or the same vector expressing normal Mot1 or the Mot1 variants shown in A from the galactose-inducible GAL1 promoter, grown to mid-exponential phase in Glc medium, shifted to Gal medium for 3 h, harvested, and lysed with glass beads. Samples of the resulting extracts (30 μg of total protein) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-c-Myc mAb 9E10. The migration positions of molecular weight markers are shown (left).

Indeed, co-overexpression of SPT15 (yTBP) from a constitutive promoter (ADH1), but not the empty vector, alleviated toxicity of overexpressed MOT1(D1408N) (28) and each of the four growth-inhibitory derivatives (Δ1090–1259, Δ802–1259, Δ1262–1867, and Δ1089–1867) (Fig. 5B).

**Mutations in the Mot1-binding Region of yTBP Relieve**
Mot1(D1408N) Toxicity—Helix 2 mutations in yTBP crippled its interaction with Mot1 in vitro (Fig. 2). Just as mutations in Mot1 that presumably eliminate its interaction with yTBP mitigated the toxic effect of Mot1(D1408N) (Fig. 5A), mutations in yTBP that prevent its interaction with Mot1 should also reduce the growth-inhibitory effect of Mot1(D1408N). To test this prediction, yeast strains were constructed in which the chromosomal SPT15 locus was deleted and replaced by wild-type yTBP (Spt15), Spt15(K133L,K138L), or Spt15(K133L,K145L), each expressed from the endogenous SPT15 promoter on a low copy number (CEN-based) plasmid. Strains carrying either spt15(K133L,K138L) or spt15(K133L,K145L) as the sole source of yTBP were temperature-sensitive for growth, as previously reported (41). All three strains were cotransformed with a multicopy plasmid overexpressing either wild-type MOT1 (Fig. 5C, left) or MOT1(D1408N) (Fig. 5C, right) from the GAL1 promoter. On Glc medium at 25 °C, all of these strains grew equivalently (data not shown). On Gal medium at 25 °C, overexpression of MOT1 had no deleterious effect on the growth, regardless of the nature of yTBP present (Fig. 5C, left). In contrast, toxicity of MOT1(D1408N) observed in cells expressing wild-type yTBP was reduced in cells expressing Spt15(K133L,K138L) and reduced quite dramatically in cells expressing Spt15(K133L,K145L). These data provide independent and compelling evidence that Mot1(D1408N) is toxic in vivo because of its ability to sequester yTBP and that Helix 2 is a critical determinant for Mot1 recognition of yTBP. Thus, the fact that deletions within the N-terminal 800 residues of Mot1(D1408N) alleviated its growth-inhibitory effect suggests that this region of Mot1 is responsible for yTBP binding. On the other hand, since yTBP is located in the nucleus (61), it was possible that derivatives of Mot1(D1408N) that are no longer growth-inhibitory are innocuous because they are unable to enter the nucleus.

Localization of Mot1 by Indirect Immunofluorescence—We examined the subcellular localization of epitope-tagged Mot1 and its deletion derivatives by indirect immunofluorescence after brief Gal induction in diploid cells (their larger size makes visualization of subcellular compartments easier) to confirm that Mot1 is a nuclear protein, consistent with its function as a transcriptional regulator. In the absence of any Myc-tagged protein, there was little (if any) background staining. Cells expressing MOT1-Myc displayed bright fluorescence that was completely congruent with the cell nucleus, revealed by co-staining the cells with the DNA-specific dye, 4',6-diamidino-2-phenylinole (DAPI) (Fig. 6). Although not as bright, an identical pattern was observed when cells expressing MOT1-Myc from its endogenous promoter on a low copy number (CEN-based) plasmid were examined (data not shown). Thus, as expected, Mot1 is a nuclear protein.

Localization of the deletion derivatives fell into three distinct classes; representative examples of each class are shown in Fig. 6. First, full-length Mot1(D1408N) and three toxic Mot1 deletions (Δ1090–1259, Δ1262–1867, and Δ1089–1867) were localized exclusively to the nucleus, like Mot1 itself (Fig. 6). Second, the fourth toxic deletion, Δ802–1259, was localized primarily in the nucleus, but cytosolic staining was also readily visible (Fig. 6). Third, all five nontoxic deletions (Δ245–491, Δ25–243, Δ1–801, Δ494–801, and Δ244–1280) were primarily, if not exclusively, in the cytosol (Fig. 6).

Residues 195–209 of Mot1 (-KKX8RRKKK-) match the consensus bipartite NLS first recognized in frog nucleoplasmin (62). Three deletion derivatives found in the cytosol (Δ25–243, and Δ1–1100) remove this sequence (Fig. 4A, NLS represented by black bar). However, two of the deletions that localized to the cytosol, Δ494–801 and Δ244–1280, retain the putative NLS (and the 494–801 region has no obvious second-
To try to narrow down the yTBP-binding region of Mot1 further (39), additional deletions were made and tested for coimmunoprecipitation with yTBP (data not shown). No loss of yTBP binding was observed for C-terminal truncations removing all residues up to 1087. A fragment corresponding to residues 1–801 bound yTBP efficiently. However, all attempts to generate smaller N-terminal deletions capable of binding yTBP failed. Removal of as few as 100 N-terminal residues totally abolished yTBP binding activity. Thus, the yTBP-binding segment of Mot1 appears large either because contact residues are noncontiguous or because the entire region is needed to form a properly folded domain.

Conservation of the TBP-binding Region of Mot1—Two orthologs of Mot1 have been identified, *Drosophila melanogaster* 89B protein (55) and human TAF172 (63, 64). The three proteins possess ~35% identity (~50% similarity) over their first 800 residues and share ~55% identity (~70% similarity) in their C-terminal catalytic (ATPase) domains. The human TAF172 exhibits biochemical properties (63, 64) rather similar to those of Mot1 (35, 36), including ATP-dependent dissociation of TBP-TATA DNA complexes and TBP-stimulated ATPase activity. 89B protein has not been characterized biochemically but is localized to the nucleus and appears to be associated with distinct loci on polytene chromosomes (55). However, when expressed in yeast, neither 89B (65) nor hTAF170 (64) can complement a mot1 mutation.

If 89B protein is a Mot1 ortholog, then it should interact with *Drosophila* TBP (dTBP). However, it has been demonstrated recently that metazoan genomes contain additional TBP-like molecules (56, 57, 66–68). In *Drosophila* one such TBP-related factor, TRF1, has intriguing parallels with 89B protein as follows: both are essential for viability; both are expressed primarily in the central nervous system and germ cells; and both colocalize at discrete loci on polytene chromosomes (55, 56, 69). Therefore, we investigated interaction between the N-terminal domain (residues 1–825) of 89B protein, expressed as a GST fusion in bacteria (Fig. 8A), and both dTBP and dTRF1, each also expressed in bacterial cells. We reproducibly observed that beads coated with GST-89B(1–825) specifically and efficiently adsorbed dTRF1 from extracts of bacterial cells, whereas GST alone, although present in much greater amounts (Fig. 8A), did not. Likewise, beads coated with GST-89B(1–825) also bound dTBP (Fig. 8B). Even though dTBP always exhibited a higher level of nonspecific interaction with GST alone than did dTRF1, increased binding of dTBP to GST-89B was reproducibly observed in each of several independent trials.

Thus, the N-terminal segment of 89B protein binds to a TBP-related factor and to dTBP itself. Likewise, it has been shown that hTAF170 can also interact physically with hTBP (63, 64). Hence, the function of the N terminus of Mot1 in yTBP binding has been evolutionarily conserved. Our findings suggest that 89B protein plays a role in dissociating dTRF1-DNA and dTBP-DNA complexes.

**DISCUSSION**

By using a coimmunoprecipitation method, we found that Mot1 and yTBP form a high affinity complex ($K_d$ ~50 nM) in the absence of DNA, ATP, or any other yeast protein. Our results explain why Mot1 was found in TBP-containing complexes isolated from yeast extracts (30, 31, 71). This $K_d$ value is in good agreement with an estimate we obtained by an independent
kinetic method (35). It has been demonstrated both in vitro (27) and in vivo (28) that Mot1 action is antagonized by the TBP-binding general transcription factor, TFIIA. In the absence of DNA, yeast TFIIA interacts with yTBP rather weakly, with an estimated $K_d$ in the 1–2 μM range (72). Hence, if Mot1 and TFIIA were present in solution at equivalent concentrations, Mot1-yTBP complexes would be greatly favored over TFIIA-yTBP complexes. However, as we have shown here and elsewhere (35), the presence of DNA does not significantly enhance the affinity of Mot1 for yTBP, whereas the $K_d$ for interaction of TFIIA with TATA element-bound TBP is estimated to be in the low nanomolar range (72, 73). Thus, when the target is yTBP bound to a TATA box, TFIIA-yTBP-DNA complexes would be greatly favored over Mot1-yTBP-DNA complexes.

Mot1(D1408N), which lacks detectable ATPase activity (28, 39), bound yTBP with the same affinity as wild-type Mot1. The fact that Mot1-yTBP complexes formed in solution in the absence or presence of ATP (or a nonhydrolyzable analog) and that Mot1(D1408N) did not display a higher affinity for yTBP than normal Mot1 suggests (albeit indirectly) that ATP binding and hydrolysis drive neither formation nor dissociation of Mot1-yTBP binary complexes. Yet, ATP hydrolysis is required for dissociation of Mot1-yTBP-DNA ternary complexes (27, 28). Therefore, energy derived from ATP binding and hydrolysis must be channeled into conformational changes in yTBP that weaken its interaction with DNA (but not its association with Mot1), in agreement with other observations we have made (35). In vivo, however, formation of Mot1-yTBP complexes is clearly reversible because even high level overexpression of Mot1 is not detectably deleterious to cells. What process or factor is responsible for competing with Mot1 for binding to yTBP?

TFIIA appears to be the best candidate for a physiologically relevant antagonist of Mot1 action for several reasons. First, we have shown that co-overexpression of TOA1 and TOA2, the genes encoding the subunits of TFIIA, partially suppresses the dominant-negative phenotype of overexpressed Mot1(D1408N) (28). Second, addition of purified TFIIA inhibits ADI (Mot1)-dependent dissociation of yTBP-DNA complexes in vitro (27). Third, TFIIA bound to TBP contacts the DNA upstream of the TATA box (73–75), which is the same segment of the DNA that Mot1 contacts when it associates with TATA box-bound yTBP (28). Fourth, we have shown here that alteration of basic residues in Helix 2, including Lys133, Lys138, and Lys145, nearly abolish yTBP association with Mot1, as judged by direct protein binding in vitro and by the resistance conferred by these mutations to the dominant-negative effects of overexpressed Mot1(D1408N) in vivo. Two of these mutants, Sp15(K133L,K138L) and Sp15(K133L,K145L), remain bound to a TATA box even in the presence of Mot1 and ATP (27). Likewise, the same yTBP mutants are impaired in their ability to associate with TFIIA (41, 49, 76), suggesting that TFIIA contacts Helix 2. Indeed, recent NMR studies performed with TFIIA-yTBP complexes containing full-length Toa1 (77) or model Toa1-derived peptides (78) have revealed that basic residues in Helix 2 of yTBP (including LysK133, Lys138, and Lys145) contribute significant contacts with TFIIA that were not observed by x-ray diffraction because the crystallographic studies were performed on complexes containing versions of Toa1 with large deletions (74, 75). Finally, a single charge-reversal mutation in yTBP, Sp15(K145E), is sufficient to prevent yTBP binding to both Mot1 and TFIIA (79). Thus, cumulative evidence suggests that binding of Mot1 and TFIIA to TATA-bound yTBP is mutually exclusive.

By using deletions and independent strategies to assess inter- action with yTBP both in vitro and in vivo, we delimited the yTBP-binding region of Mot1 to residues 1–800. In this respect, our results are in substantial agreement with the findings of Auble et al. (36), although we found no evidence that the C-terminal portion of Mot1 contributes to recognition and binding of yTBP. Our results support the conclusion that overexpression of ATPase-defective Mot1(D1408N) is toxic to cells because it forms a nonproductive complex with yTBP. Deletions that removed the ATPase domain still bound to yTBP with near-normal affinity and displayed the same growth-inhibitory phenotype. However, C-terminally truncated Mot1 derivatives that bound yTBP in vitro, like Mot1(Δ1262–1867), were less toxic than Mot1(D1408N) when overexpressed in vivo. Co-overexpression of yTBP with full-length Mot1(D1408N) does not restore a completely wild-type growth rate, whereas co-overexpression of yTBP with Mot1(Δ1262–1867) does, implying perhaps that the C-terminal domain of Mot1(D1408N) may interact with other factors. Other Mot1-interacting proteins could contribute to the observed promotor specificity of Mot1 action (24, 80, 81).

The results we obtained in vivo and in vitro demonstrated that the N-terminal 800 residues of Mot1 are necessary and sufficient for yTBP binding. Moreover, we found that evolutionarily conserved, positively charged residues on the surface of yTBP are necessary for Mot1 binding. Our observation that the N-terminal domain of 89B protein bound dTRF1 and dTBP suggested that clusters of evolutionarily conserved, surface-exposed, and negatively charged residues within the first 800 amino acids of Mot1, 89B protein, and hTAF170 might be candidates for residues involved in TBP binding. Two sequence elements in Mot1 (Asp462–Asp463–Asp464 and Asp503–Asp504–Asp505) fit these criteria and are also conserved in apparent Mot1 orthologs in Arabidopsis thaliana and Schizosaccharomyces pombe (GenBank™ accession numbers CAB71002.1 and CAB37625.1, respectively). Hence, two corresponding Mot1 derivatives, Mot1(D462A,D463A,D464A) (mot1-462) and Mot1(D503A,D504A,D505A) (MOT1-503), were constructed by site-directed mutagenesis (65, 70). Both mutant proteins were produced at the same level as normal Mot1, and Mot1–503 fully complemented a mot1Δ mutation at each of three different temperatures tested (26, 30, and 37 °C). In contrast, cells expressing Mot1–462 as the sole source of Mot1 exhibited an obvious slow-growth phenotype at all three temperatures. However, overexpression of yTBP did not rescue the slow-growth phenotype of these cells. Moreover, using the communoprecipitation assay, Mot1–462 did not display any detectable decrease in yTBP binding in vitro, compared with normal Mot1. Thus, neither of the segments of Mot1 that we selected for site-directed mutagenesis make contacts critical for yTBP binding.

Perhaps multiple redundant elements within the N-terminal domain of Mot1 contribute to its high affinity binding of yTBP. Consistent with this suggestion, analysis of the human homolog of Mot1 (TAF11170) suggests that multiple segments of the N-terminal domain participate in TBP binding. In this same regard, recent in silico analysis suggests that Mot1 contains

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5 B. F. Pugh, personal communication.

4 S. Hahn, personal communication.
eleven HEAT repeats (82) (see Fig. 4A). HEAT repeats and related helical repeat elements are clearly involved in protein-protein interactions over extended protein-protein interfaces (83). Seven of the 11 putative HEAT repeats in Mot1 fall within the first 800 residues. The mot1-462 and MOT1-503 alleles altered residues in different candidate HEAT repeats (448–479 and 492–523, respectively). Perhaps one or more of the remaining five HEAT repeats within the N-terminal region of Mot1 provides the contacts critical for yTBP recognition.

We found that Mot1 is located in the nucleus. Deletions that removed the putative NLS (62, 84) were excluded from the nucleus (summarized in Table II). Unexpectedly, however, two deletions (∆494–801 and ∆244–1280) that did not remove the NLS, but did destroy yTBP binding, were also excluded from the nucleus. These deletions may remove sequences in Mot1 responsible for regulating its nuclear localization by phosphorylation or some other post-translational modification. However, a more parsimonious interpretation of the behavior of these mutations is that nuclear localization of Mot1 requires its binding to yTBP. A requirement for yTBP binding before nuclear import could provide a simple homeostatic mechanism for maintaining the proper Mot1-yTBP ratio in the nucleus. Perhaps yTBP binding induces a conformational change that exposes the NLS in Mot1; alternatively, nuclear translocation of Mot1 may occur via “piggy-backing” on yTBP. In this regard, the importin, Kap14, plays a major role in nuclear entry of yTBP (61, 85); whether it is free yTBP or yTBP sequestered in larger complexes that enters the nucleus via this route is not known. It will be interesting to test whether kap114 mutations have any effect on the subcellular distribution of Mot1. However, because sp15A and mot1A mutants are inviable, whereas kap114A mutants are viable, both yTBP and Mot1 must have at least one other Kap114-independent route for entering the nucleus.

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