Purification and Enzymic Properties of Mot1 ATPase, 
a Regulator of Basal Transcription in the 
Yeast Saccharomyces cerevisiae

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Running Title: Purification and characterization of yeast Mot1
The 1867-residue Mot1 protein is a member of a super-family of ATPases, some of which are helicases, that interact with protein-nucleic acid assemblies. Mot1 is an essential regulator of RNA polymerase II-dependent transcription in vivo and dissociates TATA box-binding protein (TBP)-DNA complexes in vitro. Mot1-(His)₆ was purified to apparent homogeneity from yeast extracts. The preparation efficiently dissociated TBP-TATA complexes, suggesting that no other protein or cofactor is required. Mot1 behaved as a non-globular monomer in hydrodynamic studies, and no association was detected between differentially-tagged co-expressed Mot1 constructs. ATPase activity was stimulated about 10-fold by high ionic strength or alkaline pH, or by deletion of the N-terminal TBP-binding segment, suggesting that the N-terminal domain negatively regulates the C-terminal ATPase domain (Mot1C). Correspondingly, at moderate salt concentration, Mot1 ATPase (but not Mot1C) was stimulated ≥10-fold by yeast TBP, suggesting that interaction with TBP relieves a conformational constraint in Mot1. Double- or single-stranded TATA-containing DNA did not affect ATPase activity of Mot1 or Mot1C, with or without TBP. Mot1 did not exhibit detectable helicase activity in strand displacement assays using substrates with flush ends or 5’- or 3’-overhangs. Mot1-catalyzed dissociation of TBP from DNA was not prevented by a psoralen crosslink positioned immediately preceding the TATA sequence. Thus, Mot1 most likely promotes release of TBP from TATA-containing DNA by causing a structural change in TBP itself, rather than by strand unwinding.
Transcription by all three eukaryotic RNA polymerases requires TBP. For initiation by yeast Pol II, TBP binding to a TATA-sequence present in most promoters nucleates stepwise assembly of a large complex containing TBP-associated factors (TAFs) (1), termed TFIIID, as well as multiple general transcription factors and Pol II (reviewed in 2-5). In an alternative, but not mutually exclusive, model of initiation, TFIIID binding creates a platform for recruitment of a massive holoenzyme, consisting of Pol II, the SRB proteins and other cofactors (like Gal11), as well as the general transcription factors, TFIIB, TFIIF, and TFIIH (reviewed in 6-8). In both models, promoter binding by TBP is the rate-limiting step in initiation complex formation, and thus provides a physiologically relevant target for transcriptional control by both positive (9, 10) and negative (11-14) regulatory factors.

Factors that negatively regulate eukaryotic transcription utilize a wide variety of mechanisms, including quenching of activators, promoting assembly of chromatin, recruiting chromatin-binding proteins or histone-modifying enzymes, and interfering with the general transcription machinery itself. In addition, several proteins are thought to block Pol II transcription via interaction with TBP. Such regulators include Even-skipped (eve gene product), a *Drosophila* homeodomain protein (15, 16), and a general repressor, NC2 (or Dr1-DRAP1) (17-19). NC2 binds to TBP-DNA complexes and prevents the association of TFIIA and TFIIB, possibly by altering conformation of the DNA (20-24).

Another kind of negative regulator of TBP action was first identified both genetically and biochemically in the yeast *Saccharomyces cerevisiae*. A TBP-targeted repressing activity, termed ADI for “ATP-dependent inhibitor” of TATA-binding, was detected in yeast nuclear extracts (25). ADI removed TBP from the TATA element of the Adenovirus major late promoter (AdMLP) in an ATP-dependent manner. TFIIA, and to some extent TFIIB, inhibited ADI activity by competing for binding to TBP or by stabilizing the TBP-DNA complex, or both (25). It was subsequently found that ADI corresponded to the product of the *MOT1* gene (26). The *MOT1* locus had been previously identified by temperature-sensitive mutations that increased basal expression of many Pol II-specific genes (27, 28).
The MOT1 gene was isolated by complementation of the temperature-sensitive lethality of the mot1-1 allele and encodes a 1867-residue protein (calculated $M_r$ 210 kDa), which is essential for yeast cell viability (27). The C-terminal third of the polypeptide (residues 1270-1747) bears strong sequence similarity to a domain conserved in a large super-family of ATPases (reviewed in 29) and is an efficient ATPase \textit{in vitro} (26). Mutants defective in ATP hydrolysis \textit{in vitro} are non-functional \textit{in vivo} (26). This domain is characterized by seven signature motifs, including a nucleotide-binding element. Some of the members of this super-family are demonstrated DNA or RNA helicases (reviewed in 30-32). Mot1 is the founding member of a distinct class of these proteins (27), now generally referred to as the “Snf2 family” after its most well-known member, \textit{S. cerevisiae} Snf2/Swi2 (reviewed in 33, 34). The proteins in this family, which includes representatives from bacteria, yeast, \textit{Drosophila}, and man, are significantly more closely related to each other than to any other member of this ATPase super-family. Thus, Mot1 is a novel type of TBP inhibitor, one that requires the energy of ATP hydrolysis for its action.

Because of the effects of Mot1 and Snf2 on transcription and because more distantly related members of the ATPase super-family are well-characterized helicases (35-37), it was originally proposed that Mot1 (27), Snf2 (38), and other members of that family (31) might be DNA-dependent helicases. To date, however, no member of the Snf2 family has been shown to possess helicase activity. To assess the properties of Mot1 and to begin to explore the mechanism by which Mot1 removes TBP bound to a TATA-sequence, we undertook the purification of native Mot1 from yeast cells and investigated its solution structure and catalytic properties \textit{in vitro}. Here we describe this purification and analysis of Mot1, and the cumulative evidence indicating that Mot1-dependent ATP hydrolysis does not act to release TBP from DNA by a strand unwinding mechanism.
EXPERIMENTAL PROCEDURES

Materials—All reagent chemicals were purchased from Sigma or other appropriate commercial suppliers, as specified. Restriction enzymes were purchased from New England BioLabs, Boehringer-Mannheim, or other commercial sources.

Plasmid Construction—To facilitate purification of Mot1, a sequence encoding a (His)$_6$ tract was fused to the 3’ end of the MOT1 open reading frame using a simple method for precise gene fusion based on the polymerase chain reaction (PCR) (39). For this purpose, amplification was carried out under standard conditions using Taq polymerase, 10 fmol of NotI-linearized pRSMOT1 (27) as template, and primers CM7 (5’-AAA TTT CCA CCG CGG TGG ATT AAT GAT GAT GAT GAT GAT GAC CTC GTA AAG TTT TGA TGA-3’) and CM2 (5’-CTA GAC ATG GTT GAA AAT GA-3’). The resulting 670-bp PCR product was digested with BstXI and inserted into the BstXI sites of plasmid pKH6 (26), yielding pCM112, a URA3-marked 2µm DNA-containing plasmid that carries MOT1-(His)$_6$ under control of the galactose-inducible GAL1 promoter. The construction of MOT1-(His)$_6$ was verified by direct nucleotide sequence analysis of the PCR-derived DNA. The deduced carboxy-terminal sequence of Mot1-(His)$_6$ is -KTLR$^{1867}$GHHHHHHH-COOH (authentic Mot1 residues in boldface).

Large-scale Yeast Cell Growth—S. cerevisiae strain SC295 (MATa ura3-52 leu2-3,112 reg1-501 gal1 pep4-3) (40), which is both vacuolar protease-deficient and unable to impose glucose-mediated repression, was transformed with pCM112 using a lithium acetate procedure (41) and transformants were selected on synthetic-complete medium containing 2% glucose (dextrose) and lacking uracil (SCD-Ura) (42). The plasmid-bearing cells were grown to saturation in 5 ml of liquid SCD-Ura at 30°C and used to inoculate a larger culture (250 ml) of SCD-Ura + 25 mg/ml kanamycin, which was also grown to saturation and then used as the inoculum for an 110-liter culture of SCD-Ura + 25 mg/ml kanamycin in a 200-liter fermenter (New Brunswick Scientific). Cells were grown in the fermenter at 30°C with vigorous aeration and stirring to an $A_{600nm} = 0.6$, whereupon solid galactose was added to a final concentration of ~2% and the culture was incubated for additional 6 hours. Cells were then harvested using a compressed air-
driven super-centrifuge (Sharples), washed by resuspension in 500 ml of Lysis Buffer (50 mM HEPES, pH 7.8, 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA), and recollected by centrifugation for 10 min at 5000 x g. The washed cells were resuspended in Lysis Buffer (2 ml per gram of wet weight of cell paste), frozen dropwise by dripping into liquid N2, and stored at -70˚C until used.

Purification of Mot1-(His)$_6$ from *S. cerevisiae*— For a typical preparation, 270 g of frozen cell beads were thawed in a water bath at room temperature and then immediately placed on ice. All subsequent operations were performed at 4˚C. A battery of protease inhibitors were added to the cell suspension (final concentration indicated): EDTA (1 mM), phenylmethylsulfonyl fluoride (PMSF) (1 mM), benzamidine (1 mM), aprotinin (5 µg/ml), leupeptin (5 µg/ml), and pepstatin A (5 µg/ml). The cell suspension was lysed by cavitation with glass beads (0.45 mm) using ten 30-sec pulses, separated by 1-min intermittent periods of cooling, in a stainless steel Bead-Beater (Biospec Products) surrounded by an ice-water jacket. The lysate was decanted and the beads were rinsed with a small volume (100 ml) of Lysis Buffer containing 1 mM PMSF and 1 mM EDTA (Lysis Buffer + PMSF + EDTA). The lysate and rinse were pooled and clarified by centrifugation at 19,600 x g for 20 min. The supernatant fraction was decanted into fresh tubes and clarified again in the same manner. The clarified material (crude extract) was pooled and the protein concentration was determined by the dye-binding method of Bradford (43) using a commercial kit (Bio-Rad) and bovine serum albumin (BSA) as the standard. The crude extract was diluted to 15 mg/ml using Lysis Buffer + PMSF + EDTA and slowly adjusted to a final ammonium sulfate concentration at 25% of saturation by addition of the finely-ground solid salt with stirring on ice over the course of 15 min. After equilibration with stirring for an additional 30 min, the precipitated material was removed by centrifugation at 16,300 x g for 15 min. The supernatant solution was decanted to a fresh container, additional ammonium sulfate was added in a similar manner to a final concentration at 50% of saturation, and the precipitated material was again collected by centrifugation. The 25-50% precipitate was redissolved in 1 liter of Binding Buffer (50 mM HEPES, pH 7.8, 0.5 M NaCl) containing 5 mM imidazole and protease inhibitors, as above, and loaded at a flow rate of 1
ml/min onto a column containing a bed (10 ml) of Ni²⁺-iminodiacetate-agarose (His-Bind™, Novagen) that had been pre-equilibrated with Binding Buffer containing 5 mM imidazole. The flow-through was reloaded at the same flow rate and then collected. The column was washed and eluted with Binding Buffer plus increasing concentrations of imidazole in a stepwise fashion, as follows: four 20-ml washes of 5 mM imidazole, five 20-ml fractions of 20 mM imidazole, two 20-ml fractions of 40 mM imidazole, and three 10-ml fractions of 500 mM imidazole. The majority of the Mot1-(His)₆ eluted in the 40 mM and 500 mM imidazole fractions. The 40 mM and 500 mM eluates were pooled separately, placed in dialysis tubing (SpectraPor), and dialyzed exhaustively against 20 mM HEPES, pH 7.8, containing 0.5 M NaCl, and then against Buffer A (20 mM HEPES, pH 7.8, 0.5 M NaCl, 1 mM DTT, 1 mM EDTA). The dialyzates were pooled and concentrated by ultrafiltration under N₂ gas in a stirred pressure cell (Amicon) fitted with a YM30 Diaflo membrane (Amicon) to a protein concentration of 0.64 mg/ml. The protein was quantitatively precipitated by addition of ground ammonium sulfate to a final concentration at 85% of saturation and collected by centrifugation for 15 min at 17,300 x g. The pellet was redissolved in 2.5 ml of Buffer A and the insoluble particulate material was removed by centrifugation for 2 min at 14,000 rpm in a microfuge (Eppendorf). The redissolved material was loaded at a flow rate of 0.5 ml/min using an FPLC apparatus (Waters) onto a column (Hi-Prep 16/60) containing a bed of a size-exclusion resin (Sephacryl S-300HR; Pharmacia) that had been pre-equilibrated with Buffer A. The column was eluted with Buffer A and fractions (2 ml) were collected. The elution position of Mot1-(His)₆ was detected using a flow-cell, UV monitor, and chart recorder, and confirmed by SDS-PAGE followed by silver staining and by immunoblotting using specific rabbit polyclonal anti-Mot1 antibodies (26). Subsequent experiments were performed with the peak fractions eluted from the size-exclusion column, unless otherwise indicated.

Estimation of Native Molecular Mass by Gel Filtration Chromatography— Globular protein standards (Pharmacia) were purchased as lyophilized powders and resuspended individually, or as a mixture, at a concentration of 1 mg/ml each in either Buffer A or Buffer A.
containing 10 mM MgCl₂ and 1 mM ATP. The standards were loaded in a volume of 2 ml at a 
flow rate of 0.5 ml/min using an FPLC apparatus (Waters) onto a column (Hi-Prep 16/60) 
containing a bed of a size-exclusion resin (Sephacryl S-300HR; Pharmacia) that had been pre-
equilibrated with the same buffers. The column was eluted with the same buffers and 2 ml 
fractions were collected. Elution position of each protein standard was monitored both by an in-
line UV detector and by spectrophotometric analysis of corresponding fractions. Elution 
volumes of the standards were used to construct a selectivity curve (K_{av} vs. log M_r). After 
calibration with the standards in each of the two different buffers, purified Mot1-(His)_6 (Ni²⁺-
iminodiacetate-agarose eluate) was loaded onto the calibrated column in the same volume at the 
same flow rate and eluted. The fractions obtained were analyzed by SDS-PAGE with silver 
staining and by immunoblotting to determine the elution volume of Mot1-(His)_6.

Estimation of Native Molecular Mass by Rate Zonal Sedimentation — Solutions of 20, 15, 
10, and 5% sucrose (w/v) in Buffer B (20 mM HEPES, pH 7.8, 200 mM NaCl, 1 mM DTT, 0.1 
mM EDTA) were layered sequentially into upright 13 x 51mm Ultra-Clear centrifuge tubes 
(Beckman), then equilibrated at 4°C for 4 hours in a horizontal position and returned to an 
upright position, thereby generating a linear 5-20% sucrose gradient. Fractions from 
representative tubes were collected and analyzed using a refractometer to confirm the linearity of 
the gradients (data not shown). Lyophilized globular protein standards were resuspended in 
Buffer B at 1 mg/ml and mixed with purified Mot1 (Sephacryl S-300HR eluate), either 
individually, in pairs, or all together. Samples (150 µl) of the protein solution were layered on 
top of four separate gradients and then subjected to centrifugation in an SW-55Ti rotor at 50,000 
rpm (250,000 x g at r_{avg}) for 6 hr at 4°C in an ultracentrifuge (Beckman L8-80M), which was 
allowed to decelerate without braking. Fractions (100 µl) were collected from each tube using an 
automated apparatus (Model 640 Density Gradient Fractionator, ISCO) and the positions of the 
proteins were determined by UV spectrometry (for hemoglobin, \lambda_{max} = 555 nm), by protein 
assay (for catalase and aldolase), and SDS-PAGE and immunoblot analysis [for Mot1-(His)_6].
Co-expression of Differentially-Tagged Functional Variants of Mot1—Yeast strain BJ2168 (MATa ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407) (44) was transformed with 2 µm DNA-containing plasmids expressing MOT1-myc (pKH7) (45) and either MOT1-(His)6 (pCM112) or untagged MOT1 (pKH6) (45). Cells were grown in an appropriate selective medium and induced for 2 hours by the addition of galactose (2% final concentration). Cells were harvested by centrifugation, washed, and resuspended in Binding Buffer. Extracts were prepared immediately thereafter by vigorous vortex mixing with glass beads and, after clarification, were loaded in parallel onto Ni2+-iminodiacetate-agarose columns. The columns were washed with the same buffer and then eluted using the same buffer containing increasing concentrations of imidazole (20, 40, 60, and 500 mM), and then stripped with Binding Buffer + 100 mM EDTA. Proteins present in the eluted fractions were analyzed by SDS-PAGE and immunoblotting with an appropriate antibody.

Purification of Bacterially-Expressed TBP and Bacterially-Expressed Mot1C—The construction of a plasmid that expresses the catalytically-active C-terminal ATPase domain of Mot1 (Mot1C) in Escherichia coli and purification of this fragment of the enzyme from this source have been described previously (26). Likewise, full-length yeast TBP (SPT15 gene product) was expressed in E. coli and purified by a procedure described in detail elsewhere (46).

Assay of ATPase Activity—Samples of Mot1-(His)6 (765 ng) or Mot1C (700 ng) in Buffer A containing 10% glycerol, or an equal volume of the same buffer, were incubated, in duplicate, in a total volume of 50 µl containing 4 mM MgCl2, 0.1 mg/ml BSA, 0.25 mM [α-32P]ATP (20 µC/mmol), and 50 mM HEPES (and/or other buffering species adjusted to the pH value indicated in the experiments shown) with or without added salt at the concentrations indicated in the experiments shown. Reactions were initiated by the addition of enzyme, incubated for 30 min at 30°C, and terminated by addition of an equal volume of ice-cold 100 mM EDTA. Products were analyzed by thin layer chromatography and scintillation counting as described before (26). When present, yeast TBP purified from E. coli was added in the amounts indicated; likewise, when present, “TATA DNA” was added as an 28-base oligonucleotide...
containing the sequence of the TATA element of the AdMLP (5'-CCT GAA GGG GGG CTA TAA AAG GGG GTG G-3'), which was generated by chemical synthesis, and used either in single-stranded (ssDNA) or double-stranded (dsDNA) form, the latter of which was prepared by annealing to a perfectly complementary oligonucleotide.

Assays of Helicase Activity— The universal template for each substrate was a circular single-stranded phage DNA containing the complement of a TATA box. To prepare this molecule, a 101-bp EcoRI-BamHI fragment from plasmid pRW2 (47) that contains the AdMLP TATA-sequence was inserted into the corresponding sites of the replicative form of M13mp18, yielding plasmid pKH50. Progeny single-stranded phage DNA was isolated from E. coli cells infected with pKH50 using precipitation with polyethylene glycol according to standard procedures (48). A helicase substrate with a flush-ended double-helical region was prepared by annealing the circular phage DNA with the perfectly complementary 28-base “TATA DNA” oligonucleotide. Helicase substrates with 5’- and 3’-single-stranded tails were prepared by annealing the circular phage DNA with oligonucleotides “5’-TATA” and “3’-TATA”, which were otherwise identical to the “TATA DNA” oligonucleotide, except that each contained an additional 10-base, non-complementary, overhanging sequence at its 5’- or 3’-end, respectively. The oligonucleotides were labeled using two different strategies. The “TATA DNA” and “5’-TATA” oligonucleotides were labeled at their 3’-ends, as follows. The single-stranded template (1 pmol) was mixed with a 10-fold molar excess of the oligonucleotide, heated to 85˚C, and cooled slowly to room temperature on the benchtop. To the annealed substrate, 20 µCi of [α-32P]dGTP (3 Ci/µmol; NEN) and 10 units of the Klenow fragment of E. coli DNA polymerase I (New England BioLabs) were added in a buffer (60 µl total volume) containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, and 6.67 mM DTT. The mixture was incubated at room temperature for 30 min, and then reaction was terminated by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The “3’-TATA” oligonucleotide was labeled at its 5’-end using [α-32P]ATP (3 Ci/µmol; NEN) and T4 polynucleotide kinase (New England BioLabs), as recommended by the manufacturer, and then reaction was terminated by extraction with
phenol:chloroform:isoamyl alcohol (25:24:1). Thereafter, a 10-fold molar excess of the labeled
“3’-TATA” oligonucleotide was mixed with one pmol of the single-stranded template, heated to
85˚C, and cooled slowly to room temperature. All three annealed substrates were separated from
unincorporated label by gel filtration over a bed (1 ml) of Sephadex G-25 (Pharmacia) equilibrated
in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 100 mM NaCl, and then
further purified before use by centrifugation through a Microspin S-400 column (Pharmacia).

Helicase reactions (20 µl total volume) contained 0.2 mM substrate (in nucleotides), 50
mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT, 5 mM MgCl₂, and 1 mM
ATP. Reactions were initiated by the addition of enzyme, either Mot1-(His)₆ (765 ng), Mot1C
(700 ng), or, as a positive control, purified SV-40 T-antigen (500 ng; gift of E. Fouts and M.
Botchan, Univ. of California, Berkeley) in the presence or absence of purified yeast TBP (1 µg).
Reactions were incubated at 30˚C for 1 hour and terminated by addition of 2 µl of 10x Stop
Buffer (40% glycerol, 50 mM EDTA, 1% SDS, 0.1% bromophenol blue). To separate displaced
oligonucleotide from unreacted substrate, samples were resolved by electrophoresis through a 5%
non-denaturing polyacrylamide gel (48). Gels were fixed in 10% methanol-10% acetic acid, dried
under vacuum, and analyzed by autoradiography using X-ray film (Kodak Biomax-MR).

**SDS-PAGE and Immunodetection of Mot1** — Protein samples were diluted in SDS-PAGE
loading buffer, heated to 100˚C for 1 min and resolved by discontinuous SDS-PAGE on 6.5%
polyacrylamide slab gels (49). Proteins were visualized by staining with either Coomassie
Brilliant Blue dye or silver nitrate (50). For immunodetection, proteins were transferred
electrophoretically to a nitrocellulose membrane (BAS3, pore size 0.2 µm; Schleicher and Schuell
) using a Trans-Blot-SD™ apparatus (Bio-Rad), as directed by the manufacturer. The membrane
was incubated at room temperature for 1 hr in blocking buffer (5% non-fat dry milk, 500 mM
NaCl, 25 mM Tris-HCl, pH 7.5, 0.1% NP-40) and then incubated with an appropriate primary
antibody in blocking buffer for 1 hr (or, occasionally, overnight). For detection of Mot1, a
1:10,000 dilution of a rabbit polyclonal anti-Mot1 antiserum that was raised against the carboxy-
terminal 612 residues of Mot1 (45) was used. For detection of c-Myc-tagged Mot1, a 1:5,000
dilution of ascites fluid containing anti-c-Myc monoclonal antibody 9E10 was used. After thorough washing and incubation with an appropriate horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology or BAbCo), immune complexes were visualized using a commercial chemiluminescence detection system (Renaissance™, Dupont-NEN), as recommended by the manufacturer.

_Gel Mobility Shift Assay—_ A double-stranded DNA probe spanning the AdMLP was prepared by digesting pRW2 (47) with _Eco_RI (at position -50) and _Xba_I (at position +33). The resulting 83-bp fragment was end-labeled by filling in the overhangs using the Klenow fragment of _E. coli_ DNA polymerase I in the presence of [α-³²P]dATP (NEN). The radiolabeled DNA probe was separated from unincorporated radioactivity by gel filtration over a bed (1 ml) of Sephadex G-25 (Pharmacia) equilibrated in TE buffer containing 100 mM NaCl. The amount of labeled probe was quantitated by both scintillation counting and a dot-blot assay using ethidium bromide staining and a standard curve prepared with samples of DNA of known concentration (51). The concentration of the probe was ~0.5 µg/ml, which corresponded to ~7.5 fmol probe/µl with a specific activity of ~1.6 x10⁴ cpm/fmol. Proteins were mixed with 1 µl of probe-containing solution in DNA-Binding Buffer (40 mM Tris-HCl, pH 8.0, 60 mM KCl, 5 mM MgCl₂, 4% glycerol, 0.1% Brij 58, 1 mM DTT, 100 ng poly[dI-dG] (Boehringer Mannheim) and 100 µg/ml BSA) in a total volume of 20 µl. After a 30 min incubation at room temperature, either 0.25 mM ATP (pH 8.0) or an equivalent volume of pH 8.0 water was added, and incubation was continued for an additional 5 min at room temperature. The reaction mixtures were immediately loaded onto a native 6% polyacrylamide gel and separated by electrophoresis, essentially as described in detail elsewhere (25). Gels were fixed in 10% methanol-10% acetic acid, dried under vacuum, and analyzed by autoradiography using X-ray film (Kodak Biomax-MR) or quantitated using a PhosphorImager™ (Molecular Dynamics).

_Preparation of Psoralen Cross-Linked DNA—_ A sample (4 nmol) of a “short” oligonucleotide (CM6; 5'-ACC GGG TGT TCC TGA AGG TAG GCT-3’) and a sample (4 nmol) of a complementary “long” oligonucleotide (CM5; 5'-CGC GCC CCC ACC CCC TTT
TAT AGC CTA CCT TCA GGA ACA CCC GGT-3') were annealed and crosslinked (between the nucleotides indicated in boldface) using the reagent, 4'-hydroxymethyl-4,5',8-trimethylpsoralen (“psoralen”), essentially as described in detail elsewhere (52). The crosslinked oligonucleotides were 5'-end-labeled using [α-32P]ATP (Amersham) and T4-polynucleotide kinase (New England Bio-Labs), as recommended by the suppliers. Reaction was terminated by denaturation of the enzyme by incubation at 70˚C for 10 min. The short oligonucleotide was then extended by addition of excess Klenow fragment of *E. coli* DNA polymerase I and incubation in the presence of 50 µM of each of the four dNTPs. After extension, the resulting duplex sequence corresponds to the AdMLP, except for the 2-bp change made to direct the psoralen cross-link to that specific site (in boldface above). The full-length and cross-linked product was separated from all of the other material by boiling and quick-cooling on ice (the cross-linked product rapidly renatures) followed by resolution by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. The crosslinked duplexes had a distinctly slower mobility than the separate, fully-denatured constituent oligonucleotides. The band corresponding to the radiolabeled, full-length, and cross-linked probe was eluted from the gel in TE and used for gel retardation experiments, as indicated.
RESULTS

Purification of Mot1-(His)_6— Full-length Mot1 was tagged at its carboxy-terminus with a tract of six His residues to facilitate purification of the enzyme by use of Ni^{2+}-chelate affinity chromatography. When expressed in yeast from the MOT1 promoter on a low-copy-number (CEN) plasmid, the tagged protein, Mot1-(His)_6, was indistinguishable from wild-type Mot1 in its ability to rescue the viability of an otherwise lethal chromosomal mot1Δ mutation (data not shown). To further simplify purification, MOT1-(His)_6 was expressed under the transcriptional control of the strong inducible GAL1 promoter in S. cerevisiae strain SC295 (40), which carries the reg1-501 mutation that prevents glucose repression of GAL-dependent gene expression (53). As described in detail in Experimental Procedures, Mot1-(His)_6 was purified to apparent homogeneity by ammonium sulfate fractionation, Ni^{2+}-chelate affinity chromatography (Fig. 1A), and gel filtration chromatography (Fig. 1B). Identity of Mot1-(His)_6 was confirmed by immunoblotting of samples using both anti-Mot1 antiserum (45) and a commercial anti-polyHis antibody (data not shown). The yield was over 1.5 mg of purified Mot1-(His)_6 per 100 g of wet cell paste (Table I). As observed previously for Mot1 prepared by in vitro translation (26), Mot1-(His)_6 migrated with an apparent molecular mass of 175-180 kDa upon SDS-PAGE, somewhat smaller than its calculated molecular mass (210 kDa) (27). Mot1 contains several highly net negatively- and positively-charged regions; and, anomalous migration of proteins with such highly charged regions has been observed often (see, for example, 54). The purified enzyme should contain native post-translational modifications (if any) because it was isolated from yeast cells. Mot1-(His)_6 (hereafter Mot1) of ≥95% purity (Fraction IV) was used in all of the experiments presented here, unless otherwise specified. In particular, there was no detectable TBP in the purified Mot1 preparations. This result was expected because, as we have noted (45) and will describe in greater detail elsewhere^2, Mot1-TBP association is disrupted by high ionic strength and, for this reason, high salt (500 mM NaCl) was included in all buffers at every stage of the purification.
Native State of Mot1— During the gel filtration step of purification, Mot1 eluted from the Sephacryl S-300 column earlier than expected for its calculated molecular mass, suggesting that Mot1 might be larger than a monomer. Therefore, size exclusion chromatography was used analytically to obtain a more rigorous estimate of the apparent size of Mot1 in solution. Samples of purified Mot1 (Fraction III) were subjected to gel permeation chromatography on a Sephacryl S-300HR column that had been calibrated immediately beforehand. The observed elution volume ($V_e$) yielded a $K_{av}$ for Mot1 of 0.183, corresponding to a molecular mass of ~380 kDa, based on the $K_{av}$ values obtained for the four protein standards used for calibration (Fig. 2A). This result suggested that Mot1 either has a non-globular rod-like shape or undergoes a rapid monomer-dimer equilibrium in solution.

To distinguish between these possibilities, the apparent size of Mot1 in solution was also estimated by rate zonal sedimentation, using the method of Martin and Ames (55). The Stokes radius of a molecule has an opposite effect on its separation (and thus its estimated size) as judged by this method as compared to size exclusion chromatography. Samples of purified Mot1 (Fraction III) were mixed with globular protein standards of known molecular mass and subjected to centrifugation through a linear 5-20% (w/v) sucrose gradient (Fig. 2B). As assessed by immunoblotting, only one peak of Mot1 was observed (and no Mot1 was detected in any of the other fractions). Compared to the protein standards used for calibration, the sedimentation rate of Mot1 yielded an apparent molecular mass of ~160 kDa, consistent with the conclusion that Mot1 is a elongated monomeric protein (rather than a dimer). Moreover, as judged by either gel filtration or sedimentation, the hydrodynamic behavior of Mot1 was unchanged in the presence of 1 mM ATP and 4 mM MgCl$_2$ (data not shown).

Absence of appreciable homo-oligomerization of Mot1 in vivo was confirmed by co-expression of either Mot1 or Mot1-(His)$_6$ and a fully-functional Mot1 derivative tagged at its C-terminus with c-Myc epitope (Mot1-myc), followed by Ni$^{2+}$-chelate affinity chromatography of cell extracts. Both Mot1-myc and co-expressed native Mot1 were absent in the 500 mM eluate (Fig. 3, left side), demonstrating that, as expected, neither protein was retained on the Ni$^{2+}$-
chelate column. In contrast, in the extract containing Mot1-myc and co-expressed Mot1-(His)$_6$, Mot1-(His)$_6$ was found in the 500 mM eluate (Fig. 3, right side), indicating that a significant fraction of the Mot1-(His)$_6$ bound tightly to the affinity matrix, as anticipated. However, no detectable Mot1-myc was present in this Mot1-(His)$_6$-containing eluate (Fig. 3, right side). The same result was obtained with extracts prepared at salt concentrations varying from 150 to 500 mM NaCl (data not shown), indicating that Mot1 monomers do not stably interact in cell extracts. Thus, this experiment provides independent and complementary evidence that, like highly purified Mot1, native Mot1 does not self-associate. Hence, large Mot1-containing species observed by others in cell extracts, for example by sedimentation in a glycerol gradient (56), most likely represent a single molecule of Mot1 complexed with other proteins.

* Catalytic Properties of Purified Mot1— We previously expressed the carboxy-terminal third (residues 1254-1867) of Mot1 in *Escherichia coli* and purified this fragment of the enzyme (which was dubbed Mot1C) from this source (26). This segment of Mot1 contains the conserved sequence motifs shared by ATPases of the Snf2 family (27) and, as expected, was a highly active ATPase *in vitro* (26). To determine the optimal conditions for ATP hydrolysis by this catalytic domain, reactions were performed under a wide range of values of both pH and ionic strength. Mot1C exhibited maximal activity at pH 6.7 (Fig. 4A) and in the presence of 300 mM NaCl (Fig. 4B). When measured under these optimal conditions, the specific activity of Mot1C was ~330 nmol of ADP formed/min/mg.

When measured using the same assay and under the same conditions, the specific activity of purified full-length Mot1 was reproducibly quite low (~15-20 nmol/min/mg). Therefore, reactions were conducted over a broad range of pH and ionic strength values to determine the optimal conditions. Full-length Mot1 exhibited maximal activity at pH 8.5 (Fig. 4C) and in the presence of 0.8-1.2 M NaCl (Fig. 4D). A similar pH-activity profile was observed at 200 mM NaCl, but the maximal activity achieved was only ~10% of that seen at 800 mM NaCl (data not shown). Under optimal reaction conditions, the specific activity of Mot1 was in excess of 150 nmol/min/mg. Under the same conditions, the $K_m$ of full-length Mot1 for ATP was ~100 μM.
(Fig. 6A), in the same range as the $K_m$ value for Mot1C (~200 µM) determined under its optimal conditions (45).

*Effect of TBP and DNA on Mot1 Activity*— Because Mot1 in extracts is found in soluble complexes that contain TBP and other polypeptides (56-58), and because we have shown that TBP can interact with Mot1 in the absence of any other yeast proteins (45), the effect of TBP on the ATPase activity of purified Mot1 was tested. Under salt conditions optimal for ATPase activity (800 mM NaCl), addition of TBP, even in large excess, had no effect (data not shown). This result is consistent with our finding that Mot1 cannot bind TBP at ionic strengths above 300 mM NaCl (45). However, at lower salt concentrations (100-125 mM NaCl), where the ATPase activity of Mot1 alone was barely detectable, addition of TBP had a dramatic stimulatory effect on Mot1-catalyzed ATP hydrolysis (Fig. 5B). The apparent dissociation constant for the Mot1-TBP interaction averaged from five independent experiments was 100 nM. This value measured on the basis of stimulation of reaction rate is in good agreement with the $K_d$ for TBP binding to Mot1 (50 nM) determined by an independent physical method (45), as will be described elsewhere. At a molar ratio of TBP to Mot1 of 10:1, the specific activity of Mot1 was 150 nmol/min/mg, identical to that achieved by Mot1 under high salt conditions in the absence of TBP. As expected, addition of TBP had no effect on the activity of Mot1C under any condition tested (data not shown) because this fragment of Mot1 lacks the region required for TBP binding (45,59).

Addition of either a 28-nucleotide single-stranded DNA (ssDNA) or a 28-bp duplex DNA (dsDNA), which contain the AdMLP TATA sequence, up to a concentration of 7 µM (100:1 molar ratio of DNA to Mot1), had no effect on the ATPase activity of purified Mot1 (Fig. 6). Moreover, within experimental error, incubation of Mot1 with either form of DNA in the presence of TBP failed to stimulate the ATP hydrolysis activity of Mot1 above the levels seen with TBP alone. This lack of effect of DNA is consistent with the lack of DNA-binding affinity observed for Mot1 (25) (see also below).
Formation and ATP-dependent Dissociation of Mot1-TBP-DNA Complexes— The ability of highly purified Mot1 (Fraction IV) to interact with purified TBP and a radiolabeled DNA probe spanning the AdMLP TATA-sequence was examined using a gel retardation assay (60) (Fig. 7). As judged by an electrophoretic mobility shift, addition of TBP formed a readily detectable complex with the probe in either the presence or absence of ATP (lanes 2 and 3). Identity of this species as a TBP-DNA complex was confirmed by adding anti-TBP antiserum, which resulted in a marked supershift of the mobility of the complex (lane 4), whereas addition of anti-TBP antiserum to the probe alone had no detectable effect (data not shown). In contrast to TBP, addition of Mot1 alone produced no detectable complex with DNA in either the presence or absence of ATP (lanes 5 and 6). However, in the absence of ATP, the simultaneous presence of Mot1 and TBP generated a substantial amount of a Mot1-TBP-DNA ternary complex. Because the samples analyzed in lanes 2 and 7 contained identical amounts of TBP, yet the total amount of the probe shifted was far greater when Mot1 was present, it can be inferred that, in the absence of ATP, the Mot1-TBP-DNA ternary complex is more stable than a TBP-DNA binary complex. Identity of this species as the Mot1-TBP-DNA ternary complex was confirmed by addition of anti-Mot1 antiserum, which prevented formation of the complex (data not shown). As expected on the basis of prior work (25,26), in the presence of ATP, the species corresponding to both the Mot1-TBP-DNA ternary complexes and the residual TBP-DNA complexes were almost completely abolished, confirming that Mot1 acts catalytically to dissociate TBP-DNA complexes and requires the hydrolysis of ATP to do so. The fact that Mot1, purified to apparent homogeneity, efficiently generated ternary complexes with TBP-bound DNA and dissociated those complexes in an ATP-dependent manner indicates that Mot1 requires no other yeast protein or small-molecule cofactor for these activities.

Mechanism of Mot1 Action— Sequence alignments first revealed that motifs also found in certain DNA helicases are present in the Mot1 ATPase domain (27). Given that TBP binding to a TATA sequence radically alters the conformation of B-form DNA by causing a severe bend and a positive writhe (61), ATP-driven strand unwinding of promoter DNA is a plausible mechanism
by which Mot1 could catalyze the displacement of TBP from DNA. To test this possibility, both purified full-length Mot1 and purified Mot1C were tested for helicase activity using a standard strand-displacement assay (Fig. 8). For this purpose, three different substrates were constructed in which oligonucleotides corresponding to the 28-bp sequence that contains the AdMLP TATA box were bound to circular single-stranded M13 DNA containing an insert with a complementary sequence. This 28-bp sequence is identical to that in the probe used for the gel mobility shift assay (Fig. 7), in which Mot1 was able to displace TBP from the DNA in an ATP-dependent manner. In one substrate, the oligonucleotide was perfectly complementary to the insert (Fig. 8A); for the other two substrates, oligonucleotides with additional, non-complementary 10-nucleotide 3’- or 5’-overhangs were used, generating branched forms (Fig. 8B).

As a positive control, an equivalent concentration of a known DNA helicase, SV40 large T antigen (T-Ag), was assayed under the same conditions. In reactions with all three substrates, SV40 T-Ag displayed readily detectable strand displacement activity (Fig. 8A, lane 5; Fig. 8B, lanes 5 and 11), even in the absence of a ssDNA-binding protein to capture the displaced oligonucleotide. Addition of TBP inhibited the helicase activity of SV40 T-Ag (Fig. 8A, lane 6; Fig. 8B, lanes 6 and 12), suggesting that TBP binds to and stabilizes the dsDNA substrate under these conditions, preventing strand displacement. Likewise, it has been reported previously that binding of Lac repressor to its operator can block the helicase activity of SV40 T-Ag (62). In contrast to SV40 T-Ag, neither full-length Mot1 nor Mot1C displayed any detectable strand displacement, in either the absence or presence of TBP, even upon prolonged incubation. The apparent lack of helicase activity cannot be attributed to a lack of all catalytic activity in these reactions because, in parallel assays conducted under otherwise identical conditions (but containing radioactive ATP), both Mot1 and Mot1C hydrolyzed ATP and exhibited significantly greater ATPase activity than SV40 T-Ag (data not shown). Thus, if Mot1 possesses any helicase activity at all, it is decidedly less robust than that of SV40 large T antigen.
It remained possible, however, that Mot1 could cause a local unwinding of the DNA, sufficient to displace the bound TBP, but insufficient to observe wholesale strand displacement, even of a relatively short oligonucleotide. If so, such local strand separation activity might go undetected in the assay we used. To address this issue directly, a substrate was constructed that contained a covalent interstrand crosslink introduced using psoralen photochemistry. Based on the known base specificity of psoralen-induced DNA crosslinks (63, 64), and the method used to prepare the substrate (see Experimental Procedures), the crosslink was introduced at a 5’-TpA site (Fig. 9A) that falls between the TBP binding site and the nucleotides that lie in close proximity to Mot1, as judged both by their protection in footprint analysis of Mot1-TBP-DNA ternary complexes (25) and by the ability of photoactivatable nucleotide derivatives at these positions to undergo UV-induced crosslinking to Mot1 (26). Thymines in 5’-TpA sites of B-form duplex DNA are the preferential targets of psoralens (63, 64). As judged by NMR analysis, interstrand psoralen crosslinks distort the double-helix of B-form DNA only slightly (65). Consistent with this conclusion, we observed that TBP was able to bind to the cross-linked substrate we prepared as efficiently as to an identical, but non-crosslinked, probe (Fig. 9B, lanes 2 and 6), although the resulting complex displayed a slightly slower mobility, perhaps indicating that the crosslinked DNA did not bend as dramatically as non-crosslinked DNA in response to TBP binding. In addition, the complexes of cross-linked DNA and TBP were able to form ternary complexes with Mot1 with nearly the same efficiency as the non-crosslinked control DNA-TBP complexes (Fig. 9B, lanes 3 and 7). Finally, in the presence of ATP, the Mot1-TBP-DNA complexes were dissociated, as observed before (Fig. 7), regardless of whether the DNA was cross-linked or not (Fig. 9B, lanes 4 and 8). Thus, the presence of a psoralen crosslink, which should prevent localized strand separation (especially that initiated 5’ to the TATA box), did not inhibit the ability of Mot1 to dissociate TBP-DNA complexes. These data provide additional evidence against the hypothesis that ATP-driven strand separation is the mechanism by which Mot1 displaces bound TBP from the DNA.
DISCUSSION

Mot1, an essential protein in *S. cerevisiae*, regulates Pol II transcription via a novel, as yet undefined, mechanism that requires ATP hydrolysis. To investigate its catalytic properties and mode of action, we purified active full-length Mot1 from yeast to apparent homogeneity in good yield (Fig. 1) and examined its biochemical properties in vitro.

Hydrodynamic measurements (Fig. 2) indicated that, in either the presence or absence of ATP-Mg\(^{2+}\), purified Mot1 is a monomeric protein with an apparent Stokes’ radius larger than expected from its mass, suggesting that it has an elongated non-globular shape. Indeed, when viewed by negative staining in the electron microscope, Mot1 is a particle with a distinct “kidney bean” appearance. Native Mot1 in cell extracts does not self-associate, as indicated by the lack of interaction between two functional, but differentially-tagged, forms of Mot1 co-expressed *in vivo* (Fig. 3). These results are relevant to the mechanism of Mot1 action because most (although not all) DNA helicases operate as homo-oligomers (66,67), typically dimers or hexamers, and display cooperativity between subunits as they unwind the nucleic acid strands (68). Some helicases, like *E. coli* Rep, are monomers in solution, but dimerize upon binding to DNA (69). However, purified Mot1 did not display detectable DNA-binding activity (in the absence of TBP) (Fig. 7). Cruder Mot1 preparations also lack DNA-binding ability (25, 26, 59, 70).

Full-length Mot1 was optimally active at higher values of both pH and salt concentration than those required for maximum activity of Mot1C, which lacks residues 1-1253 (Fig. 4). These observations suggest that the N-terminal domain of Mot1 exerts an inhibitory effect on the C-terminal catalytic domain and that electrostatic interactions may contribute to this interaction. Three lines of evidence support this proposal. First, partial unfolding of Mot1 by low concentrations of a mild denaturant (urea) caused a ~50% stimulation of Mot1 ATPase activity under otherwise sub-optimal conditions (46). Second, addition of TBP, which binds to the N-terminus of Mot1 (45), stimulates Mot1 ATPase activity to the same extent as high pH and high salt (Fig. 5B), implying that TBP binding causes a conformational change in Mot1. Third, as shown here, under optimal conditions (high salt, high pH, or presence of TBP), ATPase activity
of full-length Mot1 approaches that of Mot1C, suggesting that, when the N-terminus is absent, the C-terminal catalytic domain is maximally active.

Taken together, these results suggest that under physiological conditions (low ionic strength and neutral pH), the N-terminus of Mot1 acts to inhibit the C-terminal ATPase domain via electrostatic interactions and that binding of TBP to the N-terminus alleviates this negative intramolecular constraint. Published observations suggest that such an auto-inhibition model may apply to Snf2: its C-terminal domain exhibited readily measurable and DNA-stimulated ATPase activity, whereas purified full-length Snf2 had no detectable activity (71). In the SWI/SNF complex, however, Snf2 displayed DNA-dependent ATPase activity (72, 73). Thus, the N-terminus of Snf2 may act as an auto-inhibitory domain and its interaction with other proteins in the SWI/SNF complex presumably overcomes this inhibition. This behavior may be general for Mot1-related proteins because all family members are large and share a common organization, with the conserved catalytic (ATPase) domain at the C-terminus and divergent N-terminal domains that presumably target each enzyme to its substrate (26).

Under optimal conditions, the $K_m$ of Mot1 for ATP (Fig. 5A) is very close to that measured for Mot1C. Hence, the stimulation of ATPase activity caused by TBP binding (Fig. 5B) cannot be explained by an increase in affinity of the enzyme for its nucleotide substrate, consistent with the proposed relief of intramolecular constraint model. Furthermore, addition of single- or double-stranded DNA (with or without a TATA sequence) had no reproducible effect (either stimulatory or inhibitory) on the ATPase activity of purified Mot1 (in either the presence or absence of TBP) (Fig. 6). These results were somewhat surprising because Mot1 appears to make contact with nucleotides in the DNA 5' of the TATA-box, as judged by both footprinting and crosslinking experiments, and requires this 5'-flanking region in order to remove bound TBP from the DNA (25, 26). Moreover, it was reported that human Mot1, hTaf172, exhibits weak intrinsic ATPase activity that is not stimulated by either TBP or DNA alone, but is stimulated by TBP-DNA complexes (74). This difference between Mot1 and hTaf172 is not understood.
Availability of a homogenous preparation of full-length Mot1 allowed us to test its DNA-binding properties in the apparent absence of any other yeast protein or small-molecule cofactor. Mot1 did not stably interact with DNA (in either the absence or presence of Mg\(^2+\)-ATP); however, in the absence of ATP, Mot1 readily formed ternary complexes with TBP bound at the TATA-sequence (Fig. 7). These results are consistent with the ability of TBP, but not DNA, to stimulate the ATP hydrolysis activity of Mot1 (Fig. 6). Although it is possible that the strong (90°) kink in TATA DNA induced by TBP binding (61, 75) is necessary to expose the contacts sites for DNA recognition by Mot1, it is more likely that Mot1 is recruited to DNA-bound TBP via protein-protein interactions. This conclusion is supported by several observations. First, Mot1 forms stable complexes with TBP in the absence of DNA both in cell extracts (56-58) and in vitro (45, 59). Second, although nucleotides 5' to the TATA box that appear to be contacted by Mot1 are required for the TBP displacement reaction, this DNA is not required for formation of Mot1-TBP-DNA ternary complexes (25). Third, as shown here, TBP-DNA complexes do not stimulate Mot1 ATPase activity any more than TBP alone (Fig. 6).

There are four plausible models to explain how Mot1 might catalyze release of TBP from DNA via an ATP-dependent mechanism (Fig. 10). First, Mot1 could act as a DNA helicase to unwind DNA near the TATA sequence, thus disrupting TBP-DNA contacts. However, as shown here (Fig. 8), Mot1 lacked detectable strand-displacement activity in either the presence or absence of TBP on three different substrates (duplex, 3'-tail and 5'-tail) under conditions where Mot1 binds TBP, is a highly active ATPase, and dissociates TBP-DNA complexes. Cruder preparations of Mot1 also reportedly lacked DNA helicase activity (25). Moreover, we found that an interstrand psoralen crosslink (Fig. 9A) specifically introduced between the DNA sites contacted by Mot1 and TBP, respectively, does not block the ability of Mot1 to dissociate TBP-DNA complexes (Fig. 9B). Because a crosslink should prevent strand unwinding, yet did not interfere with Mot1-catalyzed dissociation of bound TBP, a mechanism in which Mot1 unwinds the TATA DNA sequence from its 5' side seems unlikely. This experiment does not rule out unwinding from the 3'-side, but this possibility also seems unlikely because no contacts
between Mot1 and DNA have been observed 3’ to the TATA sequence (25, 26). Furthermore, DNA-stimulated ATP hydrolysis is a characteristic feature of known DNA helicas (66, 68), yet Mot1 ATPase activity was not stimulated by DNA and DNA did not enhance the stimulatory effect of TBP (Fig. 6). Taken together, the monomeric nature of Mot1, the lack of stimulation of Mot1 ATPase activity by DNA, the lack of strand-displacement activity, and the ability of TBP bound to cross-linked DNA to act as a substrate, provide evidence against a helicase mechanism for Mot1 action. To date, DNA helicase activity has not been detected in any other Mot1-related enzyme, including: the Snf2 ATPase domain (71); full-length Snf2 in the SWI/SNF complex (73); and, the nucleotide-excision repair protein, Cockayne Syndrome gene B product (CSB) (76), and its yeast homolog, Rad26 (77).

A second model for Mot1 activity is based on the fact that TBP has a higher affinity for a TATA sequence in a small, closed circular DNA than in linearized DNA (78) and that TBP binding causes DNA to bend and partially open (61, 75, 79). Mot1, by spanning the DNA and TBP (Fig. 10), might act as an ATP-driven molecular “lever” that straightens the DNA, thereby weakening TBP-DNA contacts. Although Mot1 contacts nucleotides 5’ of the TATA box in the 28-base AdMLP (Fig. 9A), as assessed by footprinting and crosslinking experiments (25, 26), this interaction appears to be very weak (Figs. 6 and 7). Thus, this model seems unlikely, but has not been ruled out by any direct test.

A third model proposes that ATP hydrolysis powers Mot1 to translocate processively along the DNA, pushing TBP off, like a snow plow. Such a “tracking” mechanism had been proposed for SWI/SNF and for the Snf2 family in general (33). However, a direct test for DNA tracking found no evidence for this mechanism (70).

In a fourth model, which we favor, ATP hydrolysis provides the energy to drive a conformational change in Mot1, which is transmitted via protein-protein interactions to TBP. The Mot1-induced changes in TBP conformation would then be incompatible with the binding of TBP to its TATA site. Thus, Mot1 would displace TBP in a “power stroke” similar to that of cytoskeletal motor proteins (80). In support of this model, we have found that Mot1 and TBP
form a stable complex both in solution and on the DNA, whether in the absence of nucleotide or in the presence of ADP or two different non-hydrolyzable ATP analogs (45). These findings suggest that ATP hydrolysis, not nucleotide binding per se, drives TBP dissociation. Consistent with this view, Mot1(D1408N), which displays no detectable ATPase activity in vitro and cannot complement a mot1Δ mutation in vivo (26), nonetheless binds TBP with normal affinity as judged by co-immunoprecipitation experiments (45), and Mot1(K1303A), another ATPase-defective mutant (26), forms ternary complexes with TBP-DNA with wild-type affinity (59).

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FOOTNOTES

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1The abbreviations used are: AdMLP, Adenovirus major late promoter; BSA, bovine serum albumin; dsDNA, double-stranded DNA; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; Pol II, eukaryotic RNA polymerase II; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; ssDNA, single-stranded DNA; TAFs, TBP-associated factors; T-Ag, Simian Virus 40 large T antigen; and, TBP, TATA-box binding protein.

2K.E. Hansen, J.I. Adamkewicz, W.A. Prud’homme and J. Thorner, manuscript in preparation.

3E. Fouts and J.I. Adamkewicz, unpublished results.
FIG. 1. **Purification of Mot1-(His)$_6$ from budding yeast.** *S. cerevisiae* strain SC295 harboring plasmid pCM112 was grown, induced, harvested, lysed, and clarified by centrifugation as described in Experimental Procedures. The resulting whole-cell extract (W) was precipitated with ammonium sulfate; the fraction (25-50% pellet) containing Mot1-(His)$_6$ was resuspended, dialyzed, and loaded (L) onto a bed of Ni$^{2+}$-saturated iminodiacetate-agarose in a small column. The flow-through was passed over the column again and then collected (FT). The column was washed and eluted step-wise with buffers containing the indicated concentrations (5, 20, 40 and 500 mM) of imidazole. Samples of each fraction were resolved by SDS-PAGE and visualized by staining with Coomassie blue dye (A). Molecular weight markers (MW) are indicated on the left, and the band (Mot1) corresponding to Mot1-(His)$_6$ is indicated by an arrow on the right. Four fractions of the eluate (40-#1-3 and 500-#1) from metal-chelate affinity chromatography were pooled, dialyzed, concentrated, and subjected to size exclusion chromatography. (B) Samples of the resulting eluate (fractions #22-30) were resolved by SDS-PAGE, visualized by staining with silver nitrate, and the indicated fractions were pooled.

FIG. 2. **Determination of native molecular mass of Mot1.** Purified Mot1-(His)$_6$ (Fraction III; Table I) was subjected to size exclusion chromatography (A) using an FPLC apparatus (Waters/Millipore, Model 650E) on a bed of Sephacryl S-300HR under non-denaturing conditions, as described in Experimental Procedures. For this column, $v_o = 38$ ml and $v_t = 120$ ml. Molecular mass standards [and the corresponding observed $K_{av}$ values, where $K_{av} = (v_e - v_o)/(v_t - v_o)$] were: aldolase, 158 kDa (0.280); catalase, 260 kDa (0.268); ferritin, 440 kDa (0.171); and, thyroglobulin, 669 kDa (0.067). Arrow indicates relative elution position of Mot1 ($K_{av} = 0.183$), corresponding to an apparent molecular mass of ~380 kDa. (B) Samples (10 µg) of purified Mot1 (Fraction III) were mixed with globular protein standards (100 µg), layered onto a 5-20% (w/v) linear sucrose gradient, and subjected to centrifugation. Fractions were collected and the distribution of each protein was measured as described in Experimental Procedures.
Globular protein standards were: hemoglobin, 64 kDa (squares); aldolase, 158 kDa (shaded diamonds); and, catalase, 260 kDa (circles). The peak Mot1-containing fraction is indicated by the arrow. *Inset*, samples (15 µl) of the indicated fractions were analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-Mot1 antibodies.

**FIG. 3. Mot1 does not self-associate in cell extracts.** A protease-deficient yeast strain (BJ2168), carrying a multi-copy plasmid expressing from the *GAL1* promoter Myc-epitope-tagged Mot1 (Mot1-myc), was co-transformed with another multi-copy plasmid expressing from the *GAL1* promoter either unmodified Mot1 (left panel) or Mot1-(His)$_6$ (right panel). Cells were grown in glucose (2%)-containing medium selective for both plasmids and then shifted to galactose (2%)-containing medium for 2 hr to induce protein expression. Lysates were prepared by cavitation with glass beads and loaded (L), in parallel, onto two, identical, Ni$^{2+}$-saturated iminodiacetate-agarose columns. The flow-through fractions (FT) were collected, and the columns was washed sequentially in buffers containing increasing concentrations (in mM) of imidazole: 20 (W-20); 40 (W-40); and, 60 (W-60). The columns were then eluted (EL) with buffer containing 500 mM imidazole and stripped (S) with an EDTA-containing buffer. *Top*, the indicated fractions were resolved by SDS-PAGE in an 8% gel and all Mot1-related species were detected by immunoblotting with rabbit polyclonal anti-Mot1 antibodies. *Bottom*, the same fractions were separated on a second gel, which was analyzed by immunoblotting with mouse anti-Myc monoclonal antibody 9E10 to specifically detect only Mot1-myc.

**FIG. 4. Effect of pH and salt concentration on the rate of ATP hydrolysis catalyzed by Mot1C and full-length Mot1.** Samples of purified, bacterially-expressed Mot1C (700 ng) or Mot1-(His)$_6$ purified from yeast (765 ng, Fraction IV) were incubated with [a-$^{32}$P]ATP under the conditions indicated and the rate of ADP formation measured, as described in Experimental Procedures. Values shown are the average of at least two independent determinations, each performed in duplicate, and are given as percent of the maximal activity observed. Error bars
represent the range of observed values. (A) Mot1C (at 300 mM NaCl); (B) Mot1C (at pH 6.7); (C) Mot1-(His)_6 (at 800 mM NaCl); and, (D) Mot1-(His)_6 (at pH 8.0). The scales on the ordinates of Panels B and D are different.

**FIG. 5.** Response of Mot1-catalyzed ATPase activity to increasing ATP and TBP concentration. (A) The ATP hydrolysis activity of purified Mot1-(His)_6 (765 ng; Fraction IV) was measured at pH 8 in 800 mM NaCl at the indicated ATP concentrations. To ensure nucleotide was always present as Mg$^{2+}$-ATP complex, the MgCl$_2$ concentration in each reaction mixture was in 1 mM excess of the ATP concentration (81). (B) The ATPase activity of Mot1-(His)_6 (765 ng; Fraction IV) was measured at pH 8 in 100 mM NaCl at the indicated concentrations of purified, bacterially-expressed Spt15 (full-length yeast TBP). Values shown are the average of at least three independent determinations, each performed in duplicate. Error bars represent the range of values observed.

**FIG. 6.** Effect of DNA on Mot1-catalyzed ATPase activity. The ATP hydrolysis activity of Mot1-(His)_6 (765 ng; Fraction IV), corresponding to an enzyme concentration of ~70 nM, was measured at pH 8 and 100 mM NaCl in either the absence or presence of a two-fold excess of purified yeast TBP with or without either a single-stranded or a double-stranded 28-base oligonucleotide encoding the AdMLP TATA-sequence (~7 $\mu$M), as indicated.

**FIG. 7.** Binding and ATP-dependent dissociation of TBP-DNA complexes by purified Mot1. A radiolabeled DNA probe (7.5 fmol) containing the AdMLP TATA-sequence was incubated, as indicated, in absence of any added protein or with purified TBP (0.5 pmol), either alone or with anti-TBP antiserum (1 $\mu$l), or with purified Mot1-(His)$_6$ (0.125 pmol; Fraction IV), or with both TBP and Mot1. After incubation for 30 min at room temperature, 0.25 mM ATP was either added or omitted, as indicated, and incubation continued for an additional 10 min. Reactions were terminated by cooling on ice, and reaction products were analyzed by subjecting each mixture to electrophoresis on a non-denaturing polyacrylamide gel at 4˚C and visualized by
autoradiography. Migration positions of the resulting complexes and the free probe are indicated by arrows.

FIG. 8. **Purified Mot1-(His)₆ lacks detectable helicase activity.** Purified Mot1-(His)₆ (750 ng, Fraction IV), or purified SV40 T-antigen (500 ng) as a positive control, or purified Mot1C (700 ng), were mixed with DNA substrates, prepared and labeled as described in Experimental Procedures, containing either a perfect 28-base pair duplex corresponding to the AdMLP TATA-sequence (A) or an otherwise identical substrate with either a 10-base non-complementary 5’-extension (B, left side) or a 10-base non-complementary 3’-extension (B, right side), and incubated for 1 hr at 30˚ C. Reaction products were resolved by electrophoresis of each reaction mixture on a non-denaturing polyacrylamide gel and visualized by autoradiography. As a measure of the complete dissociation of labeled oligonucleotide from the complementary single-stranded circle, a sample of each substrate was heated to 100˚ C (“boiled”).

FIG. 9. **A psoralen-induced interstrand crosslink does prevent Mot1-catalyzed dissociation of TBP-DNA complexes.** (A) DNA sequence of the probe. Short (CM6) and long (CM5) oligonucleotides were designed, so that the only 5’-TpA dinucleotide (white-on-black letters) in the resulting hybrid was situated between the regions contacted by TBP and Mot1 (brackets). After crosslinking, the full duplex was generated by extension (underlined) of the shorter oligonucleotide from its 3’-OH end using the Klenow fragment of *E. coli* DNA polymerase I in the presence of all four dNTPs. The nucleotides directly contacted by TBP, as assessed from the X-ray structures of TBP-DNA co-crystals (60, 74) are indicated (boxed). Sites that, when occupied by photoactivatable nucleotide derivatives, can form either strong (bold asterisks) or weak (plain asterisks) photo-induced crosslinks with Mot1 in Mot1-TBP-DNA ternary complexes are also shown. (B) Either the crosslinked (lanes 1-4) or non-crosslinked (lanes 5-8) DNA probes were incubated, as described in the legend to Fig. 7, with TBP alone, or with TBP and an equal molar ratio of Mot1 in the presence or absence of ATP, as indicated.
TBP-DNA complexes (1) and Mot1-TBP-DNA ternary complexes (2) are indicated by the arrows.

FIG. 10. **Models for the mechanism of action of Mot1.** In the absence of ATP, Mot1 forms a stable ternary complex with TBP bound to TATA-containing DNA. In the presence of ATP, Mot1-catalyzed nucleotide hydrolysis results in the complete dissociation of TBP from DNA. Four possible models for the mechanism by which the energy of ATP hydrolysis might be harnessed by Mot1 to displace TBP from DNA are shown. 1, strand unwinding (helicase activity); 2, DNA straightening; 3, DNA tracking; and, 4, induced conformational change in TBP. See text for details.
Table I. PURIFICATION OF Mot1

| Fraction                  | Volume (ml) | Total protein (mg) | Total activitya (units) | Specific activity (units/mg) | Yield (%) |
|---------------------------|-------------|--------------------|-------------------------|------------------------------|-----------|
| I  Crude extract          | 225         | 4070               | NDb                     | ND                           | [100]     |
| II Ammonium sulfate precipitate | 240         | 1680               | ND                      | ND                           | [100]c    |
| III Ni$^{2+}$-chelate column eluate | 60          | 34.0               | 680                     | 20                           | [75]d     |
| IV Sephacryl S-300HR eluate | 10          | 1.50               | 225                     | 150                          | 25        |

*a* Assayed by ATP hydrolysis, see Experimental Procedures.

*b* ND, not determined (due to competing ATPases).

*c* Assuming 100% recovery of total activity

*d* Recovery estimated by immunoblot analysis.
A

\[ K_{av} = 0.183 \]

Mot1
B

![Graph showing protein concentration (µg/ml) against fraction number]

- Protein concentration (µg/ml)
- Fraction Number

- Mot1 mark
- Fractions 14 to 20
|       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------|---|---|---|---|---|---|---|---|
| TBP   | + | + | + | + | + | + | + |   |
| Mot1  |   |   |   |   | + | + | + | + |
| ATP   |   |   |   |   | + | + | + | + |
| α-TBP |   |   |   |   |   |   |   | + |
A

**oligencnucleotide CM6**

5' - A C C G G T G T T C C T G A A G G T A G G C T A T A A A A G C C G G T C C C C C G C C - 3'

CM5 3' - T G C C C A C A A G G A C T T C C A C C C G A A G G G G G G C G G G G G G - 5'

* * *

region protected by TBP

region protected by TBP + Mot1

B

| ATP | + | + | + |
|-----|---|---|---|
| Mot1| + | + | + |
| TBP | + | + | + |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|

2 → 1

**crosslinked**

**non-crosslinked**
Purification and Enzymic Properties of Mot1 ATPase, a Regulator of Basal Transcription in the Yeast Saccharomyces cerevisiae
Joanne I. Adamkewicz, Christopher G.F. Mueller, Karin E. Hansen, Wendy A. Prud'homme and Jeremy Thorner

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