Two-step Mechanism for Modifier of Transcription 1 (Mot1) Enzyme-catalyzed Displacement of TATA-binding Protein (TBP) from DNA

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Background: Mot1 catalyzes ATP-dependent disruption of TBP-DNA complexes.

Results: Mot1 binding to TBP-DNA complexes induces a conformational intermediate in which DNA is unbent.

Conclusion: We propose a two-step mechanism for Mot1 in which DNA unbending is followed by ATP-dependent DNA translocation.

Significance: Mot1 is a model Sf2/Sw2 enzyme whose catalytic mechanism is relevant for understanding how other enzymes in the family function.

The TATA box binding protein (TBP) is a central component of the transcription preinitiation complex, and its occupancy at a promoter is correlated with transcription levels. The TBP-promoter DNA complex contains sharply bent DNA and its interaction lifetime is limited by the ATP-dependent TBP displacement activity of the Sf2/Sw2 ATPase Mot1. Several mechanisms for Mot1 action have been proposed, but how it catalyzes TBP removal from DNA is unknown. To better understand the Mot1 mechanism, native gel electrophoresis and FRET were used to determine how Mot1 affects the trajectory of DNA in the TBP-DNA complex. Strikingly, in the absence of ATP, Mot1 acts to unbend DNA, whereas TBP remains closely associated with the DNA in a stable Mot1-TBP-DNA ternary complex. Interestingly, and in contrast to full-length Mot1, the isolated Mot1 ATPase domain binds DNA, and its affinity for DNA is nucleotide-dependent, suggesting parallels between the Mot1 mechanism and DNA translocation-based mechanisms of chromatin remodeling enzymes. Based on these findings, a model is presented for Mot1 that links a DNA conformational change with ATP-induced DNA translocation.

The Sf2/Sw2 ATPases comprise a class of evolutionally conserved enzymes essential for a diverse array of cellular processes, including DNA repair, recombination, and transcriptional regulation (1, 2). Members of this family use the energy derived from ATP hydrolysis to alter protein-DNA interactions, and several have been shown to function as ATP-dependent DNA translocases (3–11). However, detailed mechanistic information for enzymes in this family is limited, and whether these enzymes manipulate DNA or protein substrates in ways beyond translocation is an open question. The Sf2/Sw2 ATPase Mot1 (12) uses its ATP hydrolysis activity to disrupt the TBP-DNA complex (13–16). Because Mot1 catalyzes such a simple reaction, it is an ideal model enzyme with which to explore the function of this ATPase family.

An important component of this model system is the target of Mot1, TBP. TBP is an essential component of the preinitiation complex, and through severe DNA bending, helps nucleate preinitiation complex formation (17–22). Although many eukaryotic promoters lack a canonical TBP binding site (TATA box), TBP is found at both TATA containing and TATA-less promoters (23, 24), and TBP occupancy at promoters correlates with transcriptional activity (25, 26). Because of the central role of TBP in transcription regulation, it is not surprising that it is tightly regulated in the cell. In yeast, TBP is highly mobile, and this mobility is Mot1-dependent (27). A significant proportion of the TBP pool is highly dynamic in human cells as well (28, 29). This contrasts with the lifetime of the isolated TBP-DNA complex in vitro, which is much longer (30–32). By limiting the time TBP can reside at a promoter, Mot1 appears to influence gene expression in a number of ways. Among them, the ability of Mot1 to remove TBP promotes RNA synthesis precision and ensures that an adequate pool of free TBP is available to nucleate the assembly of new preinitiation complexes (33, 34).

Although much effort has been focused on characterizing the structural organization of the Mot1-TBP-DNA complex (13, 14, 35), little is known about conformational intermediates whose characterization is critical for understanding the ATP-dependent catalytic cycle. Mot1-TBP complexes have relaxed sequence specificity for DNA (36), but the cause of this altered behavior is unclear. In contrast to other Sf2/Sw2 family members (37–41), full-length Mot1 has no detectable DNA binding affinity (16, 36, 42, 43), and the ATPase activity of Mot1 is stim-
ulated by TBP in vitro (44, 45). However, biochemical evidence indicates that the ATPase domain is in close proximity to DNA when Mot1 is assembled with TBP-DNA (35, 43), suggesting direct Mot1-DNA interaction.

In this study, we set out to better define the role and fate of DNA during the Mot1-dependent TBP-DNA displacement reaction. Using FRET and gel-based assays, we observed that the formation of the Mot1-TBP-DNA ternary complex induces DNA unbending. Prior work has shown that FRET is an accurate and sensitive measure of the extent of DNA bending in the TBP-DNA complex (46–51). Moreover, we found that in contrast to what has been observed for full-length Mot1, the isolated ATPase domain can bind directly to DNA. Although the DNA trajectory is very different in the Mot1-TBP-DNA ternary complex compared with TBP-DNA, our results suggest that in the absence of ATP, ternary complex stability arises from both Mot1-DNA and Mot1-TBP interactions, with the Mot1-DNA interactions made possible by a conformational change in Mot1 accompanied by TBP binding. Taken together, the results suggest a new model for Mot1 action in which TBP displacement results from a two-step mechanism in which the induced fit of Mot1 to TBP-DNA primes the complex for dissociation mediated by ATP-driven DNA translocation.

**EXPERIMENTAL PROCEDURES**

**Mot1 and Mot1C Purification**—Mot1 was purified as described previously (42, 44). The C-terminal domain of Mot1, consisting of amino acids 1254–1967 (Mot1C), was amplified—Mot1 was purified as described previously (42, 44). The C-terminal domain of Mot1, consisting of amino acids 1254–1967 (Mot1C), was amplified—mot1C was amplified from Saccharomyces cerevisiae, cloned into the pMCSG7 vector containing an N-terminal His tag (52) and transformed into BL21(DE3) pLysS cells (Novagen). 1 ml of overnight culture was inoculated into 1 liter of LB medium plus 100 g/ml ampicillin. 1 ml of overnight culture was inoculated into 1 liter of LB medium plus 100 μg/ml ampicillin and grown at 37 °C to an OD of 0.6. Cultures were put on ice for 5 min and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 16 °C overnight. Cells were harvested, washed once with 0.1 M NaCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 0.5 mM imidazole, 1 mM PMSF, 1 mM benzamidine) and stored at –80 °C.

All subsequent steps were conducted at 4 °C. Cells were thawed and resuspended in buffer A supplemented with protease inhibitors (Roche Applied Science; catalog no. 1873580). Cells were sonicated three times for 30 s, with a 2-min rest in between, followed by centrifugation for 40 min at 20,000 × g. The soluble lysate was directly loaded onto a column packed with 2 ml of nickel-nitrilotriacetic acid-agarose (Qiagen; catalog no. 30120) equilibrated with buffer A. The column was washed with 6 column volumes of buffer A, 6 column volumes of buffer B (20 mM Tris, pH 7.8, 0.5 mM NaCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 0.5 mM imidazole, 1 mM PMSF, 1 mM benzamidine), and 6 column volumes of buffer C (20 mM Tris-HCl, pH 7.8, 0.05 mM NaCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 40 mM imidazole, 1 mM PMSF, 1 mM benzamidine) and eluted into 0.5 ml fractions in buffer D (20 mM Tris-HCl, pH 7.8, 0.1 mM NaCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 250 mM imidazole, 1 mM PMSF, 1 mM benzamidine). Peak protein containing fractions were immediately loaded onto a Superdex 200 10/300 GL size exclusion column (GE Healthcare; catalog no. 17-5175-01) equilibrated in buffer E (20 mM Tris-HCl, pH 7.8, 0.1 mM NaCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol) and fractionated into 1-ml aliquots. Fractions containing monomer Mot1C were mixed with 200 μl of 50% glycerol, aliquoted, and flash-frozen in liquid nitrogen. Aliquots were stored at –80 °C until use.

**TBP and TBPAtto532 Purification**—Wild type TBP and mutant TBP S61C were purified as described in Ref. 53 with the following modifications: 1 mM β-mercaptoethanol was included in all buffers. An additional wash step with 60 mM imidazole was included prior to elution, thrombin digestion was conducted at 12 °C overnight, CaCl2 was omitted from buffer F, and 5 mM imidazole was included in buffer F. TBP was concentrated (using an Amicon concentrator with 10,000 molecular weight cut-off) into the final storage buffer.

For labeling of TBP S61C with Atto532, TBP S61C was washed into labeling buffer (25 mM Hepes, pH 7.3, 0.3 mM KCl, 20% glycerol, 1 mM EDTA) following the labeling reaction according to the manufacturer’s protocol. Maleimide chemistry was conducted in 1:15 protein:dye molar ratio overnight at 4 °C. After labeling, Atto532-maleimide was quenched with a large excess of β-mercaptoethanol, and free dye was separated from proteins using a PD-10 column (Bio-Rad; catalog no. 732-2010) following washing/concentrating into storage buffer (25 mM Hepes-KOH, pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.3 mM KCl) (using an Amicon concentrator with 10,000 molecular weight cut-off). Protein was flash frozen in small aliquots and stored at –80 °C.

**DNA Preparation**—Oligonucleotides were purchased from Integrated DNA Technologies. Double-stranded DNA templates were created by annealing complementary single-stranded oligonucleotides in oligonucleotide buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM EDTA) with the non-labeled oligonucleotide in 1:25–1:6 molar excess over the complementary labeled DNA oligonucleotide, heated to 94 °C and slowly cooled to 4 °C, and stored at 4 °C.

**Hexokinase Treatment of Nucleotides**—ATP and analogs were purchased from Sigma or MP Biomedicals. Commercially purchased nucleotides and ATP analogs, including ADP and AMP-PNP, are known to contain trace quantities of contaminating ATP (54). To ensure the experiments were not affected by contaminating ATP, we tested whether pretreatment of the nucleotides with hexokinase (to hydrolyze contaminating ATP) would change the observed nucleotide effect. Briefly, 10–20 mM nucleotides were incubated with hexokinase (Sigma) in the presence of 0.2 mM glucose, 5 mM MgCl2, and either 40 mM Tris-HCl, pH 7.9 or 40 mM Tris-HCl, pH 7.9, for 5 min (54). Following hexokinase treatment, ATPγS was separated from hexokinase by ultracentrifugation (Amicon Ultra-3K, Millipore) after incubation. To confirm the hydrolysis activity of hexokinase, thin layer chromatography was used to monitor both cold nucleotide migration and hydrolysis of 10 nM radiolabeled ATP spiked into a small aliquot of each hexokinase reaction. Hexokinase treatment did not affect the results for assays containing ATPγS; therefore, both pretreated and untreated ATPγS data are shown. All other nucleotides were pretreated with hexokinase prior to use in the Mot1C DNA EMSA.
Two-step Mechanism for Mot1

TBP and Mot1 EMSA—Native EMSA detecting TBP and Mot1 binding to DNA were conducted as follows: 20–μl binding reactions consisted of 5 nM radiolabeled TATA containing DNA ((top strands shown) Gap, 5′-TATCGGCCCAGGGCGCCCGCGATGGGGGCCCTATAAAAAGGCTGGGCG-3′; the bottom strands annealed to bases 1–23 and 25–45 of the top strand, respectively; 21-mer, 5′-GGCTATAAAAGGGCTGGGCG-3′) incubated with 25 nM TBP followed by 10 nM Mot1 in reaction buffer (4 mM Tris, 60 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.1 mg/ml BSA, 0.1% Nonidet P-40, and 30 mM cold non-TATA containing DNA (5′-CCGAAAGGTAGTGGACATGC-3′)). Complexes were incubated for 2 min followed by addition of nucleotide and TATA-containing competitor DNA (same as 21-mer) (to 0.1–1 mM and 50 mM final concentrations) where indicated or an equal volume of 10 mM Tris HCl, 1 mM EDTA (TE). Samples were loaded onto a 6% acrylamide/bis-acrylamide (29/1) (25 mM Tris, 190 mM glycine, 5 mM MgOAc, 1 mM DTT, 2.5% glycerol) with running buffer (25 mM Tris, 190 mM glycine, 5 mM MgOAc). Prior to loading, gels were pre-run for 1 h, buffer was recirculated, and then samples were loaded and run for ~4.5 h at 160 V at 4 °C. Following electrophoresis, gels were dried and then imaged and analyzed by Image Quant using a Storm PhosphorImager (GE Healthcare).

Mot1C EMSA—Native EMSA detecting Mot1C binding to DNA were conducted as follows: 5 nM radiolabeled 43-bp DNA (5′-ATCCCGAGGACGTTCCTCCGGGAAAGGGCTGGCCTATAAAAAGGG-3′ (top strand)) was incubated with 0–2.5 μM Mot1C in binding buffer (7 mM Tris pH 7.8, 100 mM NaCl, 5 mM MgCl2, 4% glycerol, 1 mM DTT, 0.4 mM EDTA). Nucleotides were present at 0.1–1 mM except for ADP AlFx (100 μM ADP, 100 μM AlCl3, 2.5 mM NaF) and ADP BeFx (50 μM ADP, 50 μM BeSO4, 1.25 mM NaF). Samples were run in 6% acrylamide/Bis (29:1) gels (1× TBE, 5 mM MgOAc) with running buffer (1× TBE, 5 mM MgOAc). Gels were pre-run at 4 °C for 1 h at 100 V. The buffer was then recirculated and samples were loaded and resolved at 160 V. Following electrophoresis, gels were dried and then imaged and analyzed by Image Quant using a Storm PhosphorImager (GE Healthcare).

Fluorescence Measurements—All fluorescence measurements were made in fluorescence buffer (20 mM Hepes-KOH, pH 7.9, 100 mM KOAc, 5 mM MgCl2, 5 mM DTT, 0.1 mg/ml BSA (10% ultrapure BSA, Invitrogen P2489), 3.3% glycerol) at room temperature (~23 °C) using the Gap and 21-mer DNA sequences described above. Fluorescence assays were conducted using an ISS PC1 fluorescence spectrometer and each steady-state spectra was measured over 90–130 s. TAMRA-Cy5 FRET (dual-labeled DNA FRET) samples were excited at 514.4 nm with a 514.5 nm band pass filter, and the emission spectrum was measured from 550–700 nm with a 550 cut-on filter. Atto532-Cy5 FRET (TBP-DNA FRET) samples were excited at 490 nm with a 490-nm filter, and the emission spectra measured from 520–700 nm with a 515-nm cut-on filter. Cy5 was directly excited at 590 nm, and the emission spectrum was measured from 640–700 nm. Fluorescence emission due to direct excitation of Cy5 at 488 nm was removed from the Atto532-Cy5 emission spectra by subtracting the normalized emission (normalized by the Cy5 fluorescence when excited at 590 nm) of Cy5 DNA excited at 488 nm in the absence of TBP-Atto532 at each wavelength. Anisotropy measurements were conducted with excitation of fluorescein at 490 nm with a 490 nm band-pass filter, and the emission was measured with a 515 nm cut-on filter and a 520–560 nm band-pass filter.

As a proxy for FRET efficiency (55), the proximity ratio (P) was calculated according to the equation: \( P = \frac{I_P}{I_o} \), where \( I_P \) is the intensity of the acceptor emission (650–690 nm for Cy5), and \( I_o \) is the intensity of the donor (560–600 for TAMRA and 540–580 nm for Atto532). Spectra were normalized to the total intensity. Estimation of the Förster radius (\( R_0 \)) for the Atto532-Cy5 FRET pair was calculated according to the equation \( R_0 = \frac{8.79 \times 10^{23} \phi \kappa^2 n^4 \lambda_e^6}{8 \pi\epsilon_0 m c \Delta \lambda^4} \), where \( \kappa \) is the orientation factor (assuming \( \kappa^2 = \frac{3}{2} \)), \( n \) is the index of refraction, \( \phi \) is the donor quantum yield, and \( \lambda_e \) is the spectral overlap of the donor emission and the acceptor absorption (57). We assumed \( n = 1.4 \), a value typical for biomolecules in aqueous solution (58). Using an upper limit for \( n \) of 1.5, we estimate that a change in \( n \) due to protein binding would affect the observed FRET reported here by ~10% or less (56, 58) (data not shown). For the kinetic assays, the PC1 was setup in the T configuration to simultaneously measure Atto532 and Cy5 fluorescence. Samples were excited at 490 nm with a 490 nm band-pass filter. In one direction, Atto532 emission was measured with a 515 nm cut-on filter and a 520–560 nm band-pass filter. In the other direction, Cy5 was measured using a monochrometer set at 0 to let all light pass and a 610 nm cut-off filter. The relative proximity ratio \( \frac{P}{P_0} \) is plotted versus time. The initial proximity ratio \( P_0 \) was defined as the average of first 15 s (no nucleotide and ATPγS) or the first 4 s (ATP). To calculate the observed lifetime of the TBP-DNA interaction, the data were fit to a first-order exponential decay curve \( A(t) = A_0 e^{-t/\tau(e^{-\beta t} + b)} \), where \( \tau \) is the observed lifetime of the TBP-DNA interaction, \( b \) is the baseline (defined as the last 50 data points ATP containing the Mot1-TBP-DNA sample), and \( A_0 \) is the amplitude of the curve (defined as 1 – \( b \)). Delay of sample mixing to measure- ment was ~10 s.

RESULTS

Functional DNA Template for Mot1 Activity Assays—The strategy for studying Mot1 function utilized two different FRET experiments, both of which required placing fluorophores on the DNA in close proximity to the binding site for TBP (the TATA box). A template containing a 1-nucleotide gap was employed to satisfy the requirement that there be at least 17 bp upstream of the TATA box for efficient Mot1-catalyzed TBP displacement while also allowing fluorophore placement (Fig. 1A) (42). We refer to this gap-containing duplex DNA template as “Gap DNA.” A native EMSA confirmed that the Gap DNA template supported the ATP-dependent TBP displacement activity of Mot1 (Fig. 1, B and C). In the absence of nucleotide, Mot1 did not bind detectably to DNA except when TBP was also present. The presence of TBP in the Mot1-dependent supershifted complex has been established by prior biochemical studies (27, 35, 36). Addition of ATP led to Mot1-dependent dissociation of the ternary complex (Fig. 1, B and C). As observed previously (44), addition of an ATP analog mimicking the ATP binding state, ATPγS, to the ternary complex did not support TBP displace-
mM nucleotide added where indicated (both concentrations yielded equivalent results) shown with S.E.

FIGURE 1. Gapped DNA template to study Mot1 catalyzed TBP displacement. A, schematic of the DNA template used in FRET assays. A 45-base top strand is annealed to two bottom strands that complement each half of the 45-mer. A 1-nr gap separates the two bottom strands at base 24. A TATA box (TATAAAAG) is located from base pairs 29–36. EMSA showing that the Gap-containing DNA template is functional for Mot1 to displace TBP. All concentrations reported are the final concentrations in the reaction. 5 nM 32P-labeled Gap DNA and 25 nM TBP (or buffer) are mixed and allowed to form TBP-TATA box-specific binary complexes, and then 10 nM Mot1 (or Mot1 storage buffer) is added to form ternary complexes. After 2 min, 1 mM nucleotides (or water) and 50 nM TATA containing competitor DNA are added, and the reaction is incubated at room temperature for 15 min before loading on the gel. Gels are run at 4 °C at 160 V for 4 h. D represents DNA, T-D represents TBP-DNA binary complex, M-T-D represents Mot1-TBP-DNA ternary complex, and the asterisk indicates a small fraction of non-duplex DNA. C, quantification of EMSA experiments on either a gapped or non-gapped DNA template with 0.1–1 mM nucleotide (or water) and 50 nM TATA containing competitor DNA are added, and the TBP-DNA binary complex, ( Terrorism) is located from base pairs 29–36. EMSA showing that the Gap-containing DNA template is functional for Mot1 to displace TBP. All concentrations reported are the final concentrations in the reaction. 5 nM 32P-labeled Gap DNA and 25 nM TBP (or buffer) are mixed and allowed to form TBP-TATA box-specific binary complexes, and then 10 nM Mot1 (or Mot1 storage buffer) is added to form ternary complexes. After 2 min, 1 mM nucleotides (or water) and 50 nM TATA containing competitor DNA are added, and the reaction is incubated at room temperature for 15 min before loading on the gel. Gels are run at 4 °C at 160 V for 4 h. D represents DNA, T-D represents TBP-DNA binary complex, M-T-D represents Mot1-TBP-DNA ternary complex, and the asterisk indicates a small fraction of non-duplex DNA. C, quantification of EMSA experiments on either a gapped or non-gapped DNA template with 0.1–1 mM nucleotide added where indicated (both concentrations yielded equivalent results), represented as the relative fraction of the ternary complex (normalized to the fraction of ternary complex in the absence of nucleotide) shown with S.E. Lanes in quantification are in the same order as in B.

FIGURE 2. Dual-labeled DNA FRET system detects Mot1-dependent unbending of TBP-DNA complexes. A, schematic illustration of the dual-labeled DNA FRET system. The DNA template is the same as described in the legend to Fig. 1A. The bottom strand complementary to the TATA box has a TAMRA (donor) fluorophore covalently attached to the 3′-end and a Cy5 (acceptor) fluorophore at the 5′-end. This DNA template is referred to as TGapC. B–E, emission spectra (normalized to total intensity) of 10 nM TGapC DNA and 25 nM TBP binary complexes titrated with Mot1 in the presence of no nucleotide (B), 0.1 mM ATP (C), or 1 mM ATP·yS (D). E, quantification of proximity ratio for all conditions. Shown are DNA without TBP and Mot1 (black dotted line), Mot1-TBP-DNA complex in the absence of nucleotide (red circles), in the presence of 0.1–1 mM ATP (blue triangles) or 0.1–1 mM ATP·yS (purple squares). Both concentrations yielded equivalent results. Error bars represent S.E. To guide the eye, data points were fit to a simple binding curve.

Mot1 Unbends TBP-DNA Complexes—To monitor TBP- and Mot1-dependent changes in DNA conformation, a dual-labeled DNA FRET assay was developed with TAMRA donor and Cy5 acceptor fluorophores flanking the TATA box of the Gap DNA (Fig. 2A, referred to as TGapC). FRET efficiency strongly depends on the distance between two fluorophores relative to a characteristic distance (R0) for 50% FRET transfer efficiency (55), which, for this FRET pair, is estimated to be ∼52Å (see “Experimental Procedures”). In the absence of TBP, the distance between the two fluorophores is expected to be ∼70Å, whereas upon TBP binding and DNA bending the x-ray crystallographic structure implies that the distance between the fluorophores should decrease to ∼47Å.

Titration of TGapC with increasing concentrations of TBP led to an increase in apparent FRET (supplemental Fig. 1, B and C). To distinguish true changes in FRET from changes in dye spectral properties that could potentially be caused by nearby protein binding, control titrations were conducted using DNAs labeled with TAMRA only or Cy5 only (hereafter referred to as no-FRET controls) (supplemental Fig. 1, D and E). TBP binding had no effect on Cy5 acceptor fluorescence but increased the fluorescence from the TAMRA donor. To account for the TBP-

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dependent increase in TAMRA fluorescence, the data were normalized to the total intensity of the spectra. Parallel native EMSA yielded apparent TBP binding affinities that agreed well with those obtained by dual-labeled DNA FRET (supplemental Fig. 1C and data not shown).

Titration of these TBP-TGapC DNA complexes with Mot1 in the presence of ATP resulted in a [Mot1]-dependent decrease in FRET, which approaches the FRET signal observed in the absence of TBP (Fig. 2, C and E). Complete TBP dissociation was not expected in this assay because competitor DNA was absent, thus allowing TBP rebinding (36, 43). Remarkably, experiments carried out in parallel in the absence of ATP or in the presence of ATPγS showed a similar [Mot1]-dependent decrease in FRET (Fig. 2, B, D, and E). No-FRET control experiments again confirmed that this decrease in apparent FRET is a true FRET change, not a [Mot1]- (or [TBP])-dependent change in spectral properties of either dye (supplemental Fig. 1, F and G). Because equivalent samples reveal the presence of a stable Mot1-TBP-DNA ternary complex in these conditions (Fig. 1B), it follows that the decrease in Dual-label DNA FRET observed here reflects an unbending of the DNA in an otherwise stable Mot1-TBP-DNA ternary complex. Such an unbent ternary complex has been postulated previously (36) but has not been tested experimentally.

TBP and DNA Remain in Close Proximity upon Mot1 Binding—The discovery of a stable Mot1-TBP-DNA ternary complex containing unbent DNA may indicate that TBP unbinds DNA upon Mot1 binding. Therefore, we developed a TBP-DNA FRET assay to monitor Mot1- and nucleotide-dependent changes in the TBP-DNA interaction (Fig. 3A). A fluorescent donor (Atto532) was attached to a unique cysteine residue at position 61 of TBP (59, 60), and a Cy5 acceptor was conjugated to the 3’ end of the bottom strand containing the TATA box of the Gap DNA template (referred to as 3’-Cy5-GAP). The R0 of Atto532-Cy5 is estimated to be ~65 Å (see “Experimental Procedures”). When TBPAtto532 is not bound to 3’-Cy5GAP DNA, there is no FRET, but upon complex formation, efficient FRET is expected because the fluorophores should be ~36 Å apart.

Titration of TBPAtto532 with increasing concentrations of 3’-Cy5-Gap DNA was accompanied by increasing FRET (supplemental Fig. 2, B and C). No-FRET control experiments revealed a slight increase in Atto532 emission upon titration with unlabeled DNA, but no changes in Cy5 emission upon titration with unlabeled TBP (supplemental Fig. 2, D and E). Parallel analysis using EMSA to monitor binding showed good agreement between the FRET signals and EMSA (supplemental Fig. 2C and data not shown). This confirms that the FRET system monitors TBP-DNA binding and moreover, that neither the cysteine mutations in TBP nor dye labeling significantly alter the TBP-DNA affinity (compare with supplemental Fig. 1C).

This TBP-DNA FRET system was used to determine whether Mot1 binding alters the interaction between TBP and DNA. Pre-formed TBPAtto532-3’-Cy5GAP complexes were titrated with increasing concentrations of Mot1 (Fig. 3, B–E; No-FRET controls in supplemental Fig. 2, F and G). In the absence of added nucleotide, no change in FRET was observed (Fig. 3, B and E). Addition of ATPγS also conferred little change in TBP-DNA FRET (Fig. 3, D and E). The lack of Mot1-dependent change in the TBP-DNA FRET (Fig. 3, B and E) suggests that TBP and DNA remain in close proximity upon Mot1 binding, and any conformational change between TBP and DNA due to DNA unbending does not significantly alter the distance between the fluorescent probes.

Equivalent titrations carried out in the presence of ATP revealed Mot1-dependent decreases in FRET, indicating Mot1- and nucleotide-dependent dissociation of TBP from DNA (Fig. 3, C and E) consistent with the EMSA (Fig. 1B). As with the dual-labeled DNA FRET assay, the TBP-DNA relative FRET levels do not approach those of naked DNA, again because there is no competitor DNA in these assays to prevent Mot1 displaced TBP re-binding to a new 3’-Cy5GAP DNA. As described below, addition of TATA-containing competitor DNA along with ATP showed a FRET decrease consistent with ATP-dependent TBP displacement (Fig. 3F).

Although the TBP-DNA steady-state FRET assay suggests that TBP and DNA remain in close proximity in the absence of nucleotide or in the presence of ATPγS, the EMSA in Fig. 1B suggests that the presence of ATPγS decreased the stability of the ternary complex, albeit not as efficiently as ATP. We therefore used this TBP-DNA FRET assay to measure the lifetime of the TBP-DNA interaction in the presence and absence of Mot1 and nucleotides.

To monitor the stability of the complex over time using this TBP-DNA FRET assay, pre-formed TBP-DNA or TBP-DNA-Mot1 complexes were mixed with 10-fold excess competitor TATA-containing DNA, with or without additional nucleotides, and the FRET was monitored over time (Fig. 3F; Table 1). When Mot1 was not present, TBP-DNA complexes dissociated slowly (Fig. 3F, black line), and the rate did not depend on nucleotide (Table 1 and results not shown). TBP in a pre-formed Mot1-TBP-DNA ternary complex dissociated from its DNA target even more slowly than TBP-DNA alone (Fig. 3F, red data points; Table 1). The lifetime of the ternary complex plus ATPγS was essentially the same as the lifetime of the TBP-DNA complex (Fig. 3F, purple data points; Table 1). As expected, the rate of TBP dissociation increased greatly in the presence of ATP (~65-fold) (Fig. 3F, blue data points; Table 1).

The above results establish that, in the absence of nucleotide, Mot1 binds to TBP-DNA complexes to yield a Mot1-TBP-DNA ternary complex that allows the DNA to unbend. TBP, nevertheless, must remain closely associated with the DNA in this state, because DNA unbending in the absence of nucleotide is not accompanied by a decrease in TBP-DNA FRET (Figs. 2B and 3B) or by loss of protection against digestion by DNase I (43). In the presence of ATPγS, Mot1 may undergo a conformational change in its nucleotide-binding domain, conferring somewhat decreased stability of the complex. Another interpretation is that Mot1 may be able to slowly hydrolyze ATPγS, which would also yield a decreased complex lifetime. Regardless, the binding of ATP is not sufficient for Mot1 to efficiently displace TBP from DNA. Consistent with published results (15, 16, 42) and Fig. 1B, addition of ATP allowed Mot1 to disrupt the TBP-DNA complex.
Mot1 also Unbends Short TBP-DNA Complexes—A simple explanation for the ability of Mot1 to unbend DNA is that the DNA conformation is altered by the interaction of the ATPase domain with the \( \sim 17 \)-bp DNA segment upstream of bound TBP (35, 43). To test this, dual-labeled DNA FRET was measured with 14- and 21-bp TATA-containing DNAs, which support TBP binding but were predicted to be too short for Mot1-mediated TBP-DNA dissociation. These DNA templates are referred to as “14-mer” or “21-mer” short DNA. EMSA (supplemental Fig. 3, A and B) showed that in the absence of nucleotide, Mot1 could bind to these short TBP-DNA complexes having only 4-bp upstream of the TATA box, an interaction that occurred primarily via interaction with TBP (35). Addition of

| Nucleotide       | \(-\text{Mot1} \) (\( n \)) | \(+\text{Mot1} \) (\( n \)) |
|------------------|-------------------------------|-------------------------------|
| No nucleotide    | \( 1390 \pm 170 \) (3)        | \( 13470 \pm 2330 \) (7)      |
| ATP\( \gamma \)S | \( 1550 \pm 110 \) (3)        | \( 1130 \pm 80 \) (11)        |

\( P/P_0 \) plotted is the proximity ratio at time \( t \) divided by the initial proximity ratio (average of first 15 s for no nucleotide or ATP\( \gamma \)S, otherwise the first four data points for the ATP reaction). Data were fit to a first-order exponential decay curve with fixed baseline (set to the last 50 s of the ATP containing sample) and amplitude \((1 - \text{baseline})\). Delay of sample mixing to measurement was \( -10 \) s. See Table 1 for complex lifetimes.
ATP to pre-formed Mot1-TBP-DNA complexes resulted only in loss of the ternary complex, with no apparent change in the amount of TBP-DNA complex. An independent assay, steady-state fluorescence anisotropy, also suggests that Mot1 does not displace TBP-short DNA complexes in the presence of ATP (supplemental Fig. 3, C and D). We conclude that, consistent with published results (43), Mot1 formed stable ternary complexes on both Gap (28 bp upstream of TATA box) and short (4 bp upstream of TATA box) DNAs in the absence of ATP; but Mot1 could displace TBP efficiently only from the Gap DNAs.

Having established that Mot1 formed stable Mot1-TBP-DNA ternary complexes on short DNAs, a 21-mer dual-labeled DNA FRET assay (DNA referred to as T21C) was used to ask whether Mot1 binding was accompanied by DNA unbending in these short complexes. Remarkably, binding of Mot1 to TBP complexes formed on T21C short DNA was accompanied by a progressive decrease in FRET, similar to the results obtained using the longer TGapC probe (Fig. 4, B and D). Equivalent results were obtained using the labeled 14-mer DNA (results not shown). Corresponding no-FRET control experiments (data not shown) confirmed that these changes in apparent FRET are not a consequence of changes in spectral properties of the individual fluorophores under these different conditions.

As expected, the T21C short DNA FRET level in reactions with TBP, Mot1, and ATP was equivalent to that of the TBP-DNA complex (Fig. 4, C and D), again indicating that TBP is not efficiently displaced by Mot1 from the short DNA (supplemental Fig. 3, A–D). These results suggest a two-step mechanism for Mot1-mediated displacement of TBP from DNA. In the first step, Mot1 binding induces a change in DNA conformation. ATP-dependent dissociation of TBP then requires additional DNA upstream of the TBP binding site.

**ATPase Domain of Mot1 Binds DNA**—A puzzling feature of Mot1 action is that, although evolutionary conservation establishes the ATPase domain of Mot1 as a close member of the Snf2/Swi2 ATPase family, unlike other enzymes in the family (38–41), full-length Mot1 lacks detectable affinity for naked DNA (see also Fig. 1B) (42). Moreover, although the ATPase activities of other Snf2/Swi2 family members are stimulated by DNA (37), the ATPase activity of Mot1 is stimulated by TBP and not by DNA alone (44, 45). Nevertheless, a sequence alignment with other enzyme family members reveals that many residues implicated in DNA binding are conserved in Mot1 (40), and mutational analysis indicates that at least some are functionally important for the catalytic action of Mot1 (43). Furthermore, biochemical results and structural data (35, 43) place the Mot1 ATPase domain in close proximity to the upstream DNA segment required for TBP-DNA dissociation.

The ability of Mot1 to induce DNA unbending led us to consider that Mot1 might have cryptic DNA binding activity, which is unmasked in the conformational change that allows it to stably form ternary complexes with altered DNA trajectory. To test this, recombinant C-terminal ATPase domain (Mot1C, amino acids 1254–1867) was assayed for DNA binding activity. EMSA revealed that in contrast to observations with full-length Mot1, Mot1C does indeed bind to DNA. As shown in Fig. 5A, the DNA shifted to a set of more slowly migrating species with increasing Mot1C concentration. Quantitative analysis of the binding data revealed that the ATPase domain binds to DNA with a $K_d$ of 400 nM (Fig. 5E and Table 2). Interestingly, the affinity of the ATPase domain for DNA was nucleotide-dependent. Mot1C had highest affinity for DNA in the absence of nucleotide or in the presence of 0.1 mM ATP (blue triangles). S.E. is shown for each data point. To guide the eye, data points for Mot1 titration without nucleotide were fit to a simple binding curve.

**DISCUSSION**

**Discovery of Unbent Mot1-TBP-DNA Ternary Complex**—This work provides the first evidence that the stable Mot1-TBP-DNA complex contains unbent DNA, even though TBP and DNA remain in close proximity (Figs. 2, B and E, and 3, B and E). This unbending signature for the ternary complex is an
Mot1C complex.

concentration was 0.1–1 mM for ATP, 1 mM for ATP
AlF₄.

Error bars

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Swi2 family member, breaks histone DNA contacts in the absence of nucleotide, facilitating the movement of DNA (61). This suggests that this behavior is a general feature of enzymes in the family. An alternative possibility is that DNA unbending is required to generate a conformation in which the ATPase can properly engage DNA for productive motor activity.

Mot1 Binding-induced DNA Unbending Independent of Upstream DNA—The results presented here are consistent with a broad literature establishing that upstream DNA is essential for efficient Mot1-mediated TBP-DNA dissociation (14). However, EMSA, anisotropy, and DNA bending FRET assays using DNA with only 4 base pairs upstream of the TATA box show that Mot1-TBP-DNA ternary complexes can form in the absence of ATP (Fig. 4, B and D, and supplemental Fig. 3, A, B, and D) (36). This is consistent with interactions between Mot1 and TBP providing the main stabilizing force for the ternary complex (35). Strikingly, DNA unbending occurred in complexes formed using short DNAs, indicating that upstream DNA is not required for this DNA conformational change. This suggests that DNA unbending is a consequence of an interaction between Mot1 and the major groove of TATA DNA, the face of DNA opposite the contact surface with TBP (44). Alternatively, DNA unbending could be a consequence of a conformational change in TBP itself induced by Mot1 binding (36). As the Mot1 NTD-TBP co-crystal does not reveal any significant change in TBP structure compared with TBP alone (35), such an effect on the DNA binding activity of TBP apparently would occur via a more subtle allosteric mechanism (14, 62–65).

Direct DNA Binding by ATPase Domain of Mot1—Demonstration that the ATPase domain of Mot1 binds to DNA with sub micromolar affinity (Fig. 5A) implicates direct Mot1-DNA interactions in contributing to the stability of the Mot1-TBP-DNA ternary complex in the absence of nucleotide. The measured affinity for the Mot1C-DNA interaction described here is similar to that observed for the ATPase domain of another Snf2/Swi2 family member, SsoRad54 (41). Together, sequence conservation, DNase I footprinting experiments (43), structural studies of the Mot1-TBP complex (35) and the direct observation of DNA binding activity of this study for Mot1C provide strong support for positioning the ATPase domain of Mot1 on DNA upstream of the TATA box.

Evidence that the ATPase activity of full-length Mot1 is stimulated by TBP (but not DNA alone) (44, 45), along with the DNA binding activity of Mot1C demonstrated here further supports the hypothesis that the N-terminal TBP-binding domain of Mot1 can sequester the ATPase domain to prevent its interaction with DNA (45). Alternatively, the lack of observed direct DNA binding of full-length Mot1 could be explained by the relatively low concentration (compared with the measured affinity of Mot1C for DNA) of full-length Mot1 typically used in DNA binding assays. If this were the case, the interaction between the N terminus and TBP of Mot1 would facilitate the Mot1-DNA interaction by increasing the local concentration of DNA near the ATPase domain of Mot1.

Nucleotide Effects on Mot1-TBP-DNA Ternary Complex—Members of the Snf2/Swi2 ATPase family are thought to use ATP binding and hydrolysis to translocate along DNA while altering protein-DNA interactions (4, 5). The crystal structure

ensemble measurement that may indicate partial DNA unbending in the complex and/or differences in the extent or dynamics of DNA bending across the population of molecules. We also demonstrate that the conserved Snf2/Swi2 ATPase domain of Mot1 directly binds DNA, linking the Mot1 mechanism to those of other Snf2/Swi2 family members (Fig. 5, A and E). These results provide evidence for a two-step model for the Mot1 catalytic mechanism, which we suggest is relevant for understanding Snf2/Swi2 ATPases in general. We propose that alteration of TBP-DNA contacts by Mot1 is necessary to subsequently move TBP off its binding site. RSC, another Snf2/

FIGURE 5. ATPase domain of Mot1 binds DNA with nucleotide-dependent affinity. EMSA showing 5 nM ³²P-labeled 43 bp DNA titrated with Mot1C in the presence of no nucleotide (A), 1 mM ATP (B), 1 mM ATPγS (C), or 0.1 mM ADP aluminum fluoride (AlF₄) (D). D represents DNA, D-M represents the DNA-Mot1C complex. E, quantification of fraction DNA bound to Mot1. Nucleotide concentration was 0.1–1 mM for ATP, 1 mM for ATPγS, and 0.1 mM for ADP AlF₄. Error bars represent the S.E. Kₛ values calculated from these data are reported in Table 2. Note that the data were best fit with a Hill coefficient of 2. Biochemical and structural data indicate that Mot1 has a single ATP binding site and functions as a monomer (35, 45), so the significance of the Hill coefficient is unclear.

TABLE 2

| Nucleotide  | Kₛ (nM) |
|-------------|---------|
| No nucleotide | 400 ± 30 (15) |
| 0.1–1 mM ATP | 2890 ± 1090 (7) |
| 1 mM ATPγS  | 2720 ± 100 (3) |
| 1 mM ADP    | 900 ± 50 (3) |
| 0.1–1 mM AMP PCP | 310 ± 80 (5) |
| 0.1–1 mM AMP PNP | 320 ± 20 (5) |
| 0.1 mM ADP AlF₄ | 2150 ± 930 (4) |
| 0.05 mM ADP BeF₄ | 2190 ± 650 (4) |

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of the SsoRad54 ATPase reveals that the Snf2/Swi2 ATPase domain is composed of two RecA-like motifs, with DNA binding along the interface between these two motifs (40, 66). Translocation along DNA would then occur by the alternating affinity of the RecA-like domains for DNA while moving unidirectionally along DNA during a round of ATP hydrolysis.

The observation that the affinity of Mot1 for DNA is modulated by nucleotides suggests that Mot1 also translocates along DNA during ATP binding and hydrolysis (Fig. 5, B–E, and supplemental Fig. 4, A–E), although the distances involved are not long (67). Demonstration that the affinity of Mot1 for DNA is nucleotide-dependent also points to a unified mechanism of nucleic acid translocation for Mot1 with the rest of the Snf2/Swi2 ATPase family (14).

**Mechanism of Mot1 Catalyzed Displacement of TBP from DNA**—Taken together, we propose the model for Mot1 action shown in Fig. 6. The first step is the formation of the TBP-DNA complex (step 1) (Fig. 1B and supplemental Fig. 2B), which contains bent DNA (supplemental Fig. 1B) (17, 18). Upon binding of Mot1 to the TBP-DNA complex, the TBP-DNA interaction is altered, resulting in DNA unbending (step 2) (Figs. 2B and 4B). This Mot1-TBP-DNA ternary complex is more stable than the binary TBP-DNA complex (Fig. 1B), reflecting direct interactions between all three components. TBP remains closely associated with the DNA in this state because DNA unbending is not accompanied by a decrease in TBP-DNA FRET (Fig. 3B), or by loss of protection against digestion by DNase I (43).

Upon ATP binding (step 3), the ATPase domain of Mot1 is proposed to undergo a conformational change (41), resulting in a decrease in affinity for DNA (Fig. 5C). This conformational change may destabilize the complex compared with the absence of nucleotide as revealed in the EMSA (Fig. 1, B and C) and the TBP-DNA FRET kinetics assay (Fig. 3F). However, this conformational change is not sufficient to catalyze rapid displacement of the ternary complex (Figs. 1, B and C, and 3F). During or after ATP hydrolysis (or multiple ATP hydrolysis steps), we propose that a second conformational change occurs (step 4) as Mot1 translocates along DNA due to or accompanied by changing affinity of the ATPase domain for DNA as TBP is pulled or pushed off its binding site (Fig. 5, B–E, and supplemental Fig. 4, A–E). Given the millimolar concentrations of ATP in the yeast cell (68) and the $K_m$ of Mot1 for ATP ($\sim \mu M$) (36, 43, 45), one could suppose that Mot1 is typically bound to ATP in vivo. Therefore, in the cell, Mot1 might contact TBP-DNA complexes as Mot1-ATP (step 3). After the Mot1-catalyzed displacement of TBP from DNA, all three components dissociate from one another (36) or alternatively, there may be a direct hand-off of TBP from Mot1 to another transcription factor or complex such as TFIIID or SAGA.

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