Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism

David T. Auble,¹ Karin E. Hansen,² Chris G.F. Mueller,² William S. Lane,³ Jeremy Thorner,² and Steven Hahn¹

¹Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 USA; ²Division of Biochemistry and Molecular Biology, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720 USA; ³Harvard Microchemistry Facility, Harvard University, Cambridge, Massachusetts 02138 USA

Basal transcription of many genes in yeast is repressed by Mot1, an essential protein which is a member of the Snf2/Swi2 family of conserved nuclear factors. ADI is an ATP-dependent inhibitor of TATA-binding protein (TBP) binding to DNA that inhibits transcription in vitro. Here we demonstrate that ADI is encoded by the *MOT1* gene. Mutation of *MOT1* abolishes ADI activity and derepresses basal transcription in vitro and in vivo. Recombinant Mot1 removes TBP from DNA and Mot1 contains an ATPase activity which is essential for its function. Genetic interactions between Mot1 and TBP indicate that their functions are interlinked in vivo. These results provide a general model for understanding the mechanism of action of a large family of nuclear factors involved in processes such as transcription and DNA repair.

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Transcription by RNA polymerase II (pol II) requires a set of at least 6 general transcription factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH) in addition to the multi-subunit pol II enzyme itself (Zawel and Reinberg 1993). The first step in transcription complex assembly is thought to involve the binding of TFIID to a TATA box located upstream from the start site of many pol II promoters (Van Dyke et al. 1988; Buratowski et al. 1989; Cortes et al. 1992). The TFIID-DNA complex nucleates the assembly of the remaining general factors and pol II (Maldonado et al. 1990; Cortes et al. 1992; Flores et al. 1992). TFIID from animal cells consists of an evolutionarily conserved TATA-binding protein (TBP) tightly associated with a set of polypeptides (TBP-associated factors or TAFs), at least some of which are involved in mediating the response of the general transcription apparatus to activators (Dymlacht et al. 1991; Takada et al. 1992; Goodrich et al. 1993; Hoey et al. 1993; Kokubo et al. 1994). TAFs are also found in association with yeast TBP (Poon and Weil 1993). TFIIA is unusual because its requirement for transcription in vitro depends on the particular transcription system used (Cortes et al. 1992; Ranish et al. 1992; Sayre et al. 1992; Tyree et al. 1993). One interpretation of this finding is that TFIIA is a regulatory factor whose function is necessary to counteract the action of transcriptional repressors. The requirement

for TFIIA in vitro would depend then on whether or not such transcriptional repressors are present.

Fractionation of extracts from human cells led to the identification of several factors that can repress transcription in vitro (Drapkin et al. 1993). Dr1 binds to TBP and thereby inhibits both basal and activated transcription by preventing the interaction of TFIIA and TFIIB with TFIID (Inostroza et al. 1992). Dr2 (PC3, topoisomerase I) also interacts with TBP and represses basal transcription; its inhibitory activity can be overcome by TFIIA or by transcriptional activators (Kretzschmar et al. 1993; Merino et al. 1993). Similarly, NC1 and NC2 are factors that can form complexes with TBP and prevent the interaction of TFIIA and TFIIB with TBP-DNA complexes (Meisterernst and Roeder 1991; Meisterernst et al. 1991).

Factors that globally repress pol II transcription have also been identified by genetic approaches in yeast. The four *NOT* genes (*NOT1-4*) and the *SUD1* gene were identified by mutations which increase basal expression of many genes in yeast (Collart and Struhl 1993, 1994; Yamashita 1993). The *TSF1-6* genes were also identified as repressors which may affect the basal pol II machinery (Chen et al. 1993). Genetic and biochemical data suggest that all of these proteins define pathways for repressing transcription, although the mechanism of action of these factors is unknown.

Using a similar genetic screen for increased basal transcription, the yeast *MOT1* gene was identified by mutations that led to increased basal expression of many genes (Davis et al. 1992; Piatti et al. 1992). *MOT1* is an essential gene and a recessive temperature-sensitive mutation (*mot1-1*) leads to increased basal expression of many genes in yeast at the permissive temperature (Davis et al. 1992). In contrast, the *mot1-1* allele has comparatively little effect on highly expressed genes. *MOT1* was cloned and shown to encode a large protein with sequence similarity to a family of putative helicases (Davis et al. 1992). Because loss of *MOT1* function leads to increased basal transcription, the normal role of Mot1 is presumably to repress basal transcription, perhaps by employing an ATP-dependent mechanism to remove specific proteins from DNA (Davis et al. 1992).

Using a biochemical approach, an inhibitor of TBP binding to DNA was identified in yeast (Auble and Hahn 1993). This factor, termed ADI for ATP-dependent inhibitor, was shown to specifically dissociate TBP-DNA complexes in an ATP-dependent manner. The effect of ADI is not promoter-specific, but ADI interacts with TBP-DNA complexes in a stereospecific manner. Dissociation of TBP-DNA complexes by ADI can be blocked by TFIIA and, to a lesser extent, by TFIIB. ADI can inhibit transcription in vitro, and the inhibitory effect on transcription can also be blocked by TFIIA. Thus, ADI is a repressor of pol II transcription that functions by re-

moving TBP from DNA. To further characterize ADI, the ADI polypeptide was purified and amino acid sequence was used to identify the gene encoding it. In this paper we describe experiments which demonstrate that ADI is encoded by *MOT1*, and that the ATPase activity of Mot1 is required for both its activity in vitro and its function in vivo.

Results

Identification of the ADI polypeptide

Previous work suggested that ADI specifically recognizes TBP-DNA complexes by interaction with particular residues on the outer surface of TBP, and also by interacting with DNA immediately upstream of the TATA sequence (Auble and Hahn 1993). To identify the polypeptide responsible for ADI activity, DNA probes were constructed with photoreactive nucleotides placed in the region of the promoter presumed to be in close proximity to ADI when bound to TBP-DNA complexes. Two probes were designed which contain the photoreactive nucleotides placed at slightly different positions (Fig. 1A, marked by u). The probes were synthesized to contain radiolabeled cytosine residues (marked by asterisks) immediately adjacent to the photoreactive nucleotides to tag any crosslinked proteins with a radiolabel. These experiments were performed using the conserved

Figure 1. Photocrosslinking of the ADI polypeptide to DNA. (A) The sequences of the two DNA probes used for these experiments are shown. The promoters are variants of the adenovirus major late promoter; the TATA sequence is underlined and the region protected from nuclease digestion by TBP is shown by the brackets. The probes include the photocrosslinking nucleotide, 5-[N-(p-azidobenzoyl)-3-aminoallyl]-dUMP (denoted as "u"), and adjacent radiolabeled cytosines (asterisks). These bases were positioned in the region of the promoter which is protected from nuclease digestion by ADI when it is bound to a TBP-DNA complex. (B) The DNA probes (as indicated) were incubated with: ADI; ADI plus TBP_c (the conserved carboxy-terminal domain of yeast TBP), or ADI, TBP_c, and ATP. Following a 25 min incubation in binding buffer, the reactions were treated with UV light and then either loaded directly onto a 6% nondenaturing gel (not shown) or nuclease-treated and loaded onto a 6% SDS gel. Incubation of ADI with TBP_c-DNA complexes results in the specific crosslinking of a polypeptide of 175 kD apparent molecular mass to the labeled DNA (indicated by the arrow). The sizes (kD) of radiolabeled protein size markers are shown (left).

carboxy-terminal 180 amino acids of yeast TBP (TBP_c) because ADI binds to TBP_c more tightly than to full-length TBP in the absence of ATP (Auble and Hahn 1993). Incubation of TBP_c and partially purified ADI (Auble and Hahn 1993) with either of the two radiolabeled photoprobes resulted in the formation of both TBP_c-DNA complexes and ADI-TBP_c-DNA ternary complexes which were detected by a gel mobility retardation assay (data not shown); upon addition of ATP, the amount of the TBP_c-DNA complexes was decreased and all of the ADI-TBP_c-DNA complexes were eliminated, as expected. After UV photocrosslinking, the products were treated with nucleases which digested the bulk of the DNA, except for short radiolabeled tags which were crosslinked to the protein. Analysis of these tagged proteins is shown in Figure 1B. Two polypeptides (~70 and 150 kD) were crosslinked to the DNA whether or not TBP_c or ATP was present in the reaction, indicating that these proteins are unrelated to ADI. In contrast, a polypeptide with an apparent molecular mass of 175 kD was crosslinked to each of the DNA probes, but only in the presence of TBP_c. Furthermore, the appearance of the 175-kD band was completely abolished if ATP was added prior to photocrosslinking. Because the interaction of this polypeptide with promoter DNA occurred in a manner consistent with the known behavior of ADI, this result strongly suggests that the 175-kD polypeptide is a component of ADI. In further support of this conclusion, when column profiles from the later stages of ADI purifications were examined by SDS-PAGE and silver staining, the level of ADI activity detected correlated with the amount of a 175-kD polypeptide present in the same fractions (data not shown).

Approximately 20 µg of the 175-kD polypeptide were

purified from 1.4 kg of yeast cells as described in Materials and methods. After transfer to a PVDF membrane, the protein was enzymatically digested and the sequences of two derived peptides were determined unambiguously (see Materials and methods). The two peptide sequences, NTPKHPVDITESSV and NMTNSAASLATS-PTSNQLNPK, were found to match exactly the deduced sequence of yeast Mot1 from amino acids 214–227 and 236–256, respectively.

Mot1 has several features expected of the ADI polypeptide (Davis et al. 1992; Piatti et al. 1992). First, Mot1 was found to be an essential protein which represses the transcription of many genes in yeast. Second, Mot1 is large (1867 amino acids) and contains consensus motifs for ATP binding and hydrolysis, which are embedded in a larger conserved domain of about 55 kD (Fig. 2). Mot1 is highly related to a conserved family of proteins (the Snf2/Swi2 family) which participate in activation of transcription, DNA repair, and chromosome segregation (Fig. 2). Members of this family are present in organisms from bacteria to humans (Davis et al. 1992; Bork and Koonin 1993; Henikoff 1993). Comparison of homologous blocks of sequence from any two family members indicates that they generally share between 30 and 60% amino acid sequence identity. The presence of this large evolutionarily conserved domain in all of these proteins suggests that it provides an ATP-dependent enzymatic function that is employed for many diverse purposes in cells (see Discussion).

ADI is encoded by MOT1

Nuclear extracts were prepared from wild-type *MOT1* and temperature-sensitive *mot1-1* cells. These extracts

Figure 2. Mot1 is a member of the Snf2/Swi2 family of nuclear regulatory factors. A schematic alignment of Mot1 and several related proteins is shown. The length of each protein (number of amino acids) is shown on the right. Open boxes indicate nonconserved regions; filled boxes represent conserved sequence motifs which each share between 30 and 60% amino acid identity between any pair of the proteins listed. Motifs involved in ATP binding and hydrolysis are bracketed; the K1303A mutation is located in the amino-terminal motif and the D1408N mutation is located in the carboxy-terminal motif (see text). *SNF2/SWI2* encodes an activator of transcription of many genes in yeast (Laurent et al. 1991). *hSNF2L* is thought to be a human homolog of *Snf2/Swi2* (Okabe et al. 1992). *Brahma (brm)* encodes an activator of homeotic genes in *Drosophila* (Tamkun et al. 1992). *ERCC6* (also known as *CSBC*) is a human gene involved in preferential repair of transcribed genes (Toelstra et al. 1992). *RAD5* (Johnson et al. 1992), *RAD16* (Bang et al. 1992), and *RAD54* (Emery et al. 1993) are yeast genes involved in DNA repair. *Drosophila lodestar* encodes a function required for chromosome segregation (Girdham and Glover 1991). *Etl-1* (Soininen et al. 1992) encodes a mouse developmentally-expressed protein, and *STH1* (Laurent et al. 1992) is an essential yeast gene; the functions of both *Etl-1* and *Sth1* are unknown. *HepA* is a gene from *E. coli* of unknown function (Lewis et al. 1992; Bork and Koonin 1993). The distance of 174 residues between two of the conserved motifs in *HepA* is greater than the distances between these homologous blocks in any of the other proteins analyzed, and is depicted as a bracket above the relevant region of *HepA*.

were fractionated and tested for ADI activity in a gel mobility retardation assay. As shown in Figure 3A (lanes 1,2), an extract from wild-type cells contains ADI as expected. In the absence of ATP, ADI binds to TBP–DNA complexes to produce ADI–TBP–DNA ternary complexes. In the presence of ATP, both TBP–DNA and ADI–TBP–DNA complexes are disrupted. In contrast, an extract from *mot1-1* cells prepared in parallel with the wild-type extract contains no detectable ADI activity (lanes 3,4). In the absence of ATP, no ADI–TBP–DNA complexes are formed with the *mot1-1* fractions, and in the presence of ATP, TBP–DNA complexes are not disrupted. Thus, even at the permissive temperature, extracts from *mot1-1* cells contained no detectable ADI activity, suggesting that a functional *MOT1* gene is necessary for ADI activity.

To determine if Mot1 is sufficient for ADI activity, the *MOT1* coding sequence was introduced into a plasmid, and Mot1 was produced by in vitro transcription and translation. As controls, the in vitro transcription and translation reactions were performed with no added DNA template or with a mutant *mot1* gene containing a single amino acid change (K1303A). This residue lies within a sequence motif (GxGKT) thought to be important for ATP binding and hydrolysis (Fig. 2) because substitution of the equivalent residue in Snf2/Swi2 and Rad3 has been reported to abolish their ATPase activity, but not their ability to bind ATP (Sung et al. 1988; Laurent et al. 1993).

The in vitro transcription/translation reactions contained ATP which could not be removed from the reaction products so the gel mobility retardation assays using the Mot1 proteins produced in vitro contained ATP. TBP–DNA complexes were reduced about twofold upon addition of unprogrammed lysate to a binding reaction (Fig. 3B, lanes 1,2). Strikingly, however, addition of the normal Mot1 produced by in vitro transcription and translation resulted in the complete disappearance of the TBP–DNA complexes (Fig. 3B, lane 3). The activity of

the added Mot1 is dependent on an intact ATP-binding sequence because TBP–DNA complexes are only weakly affected by Mot1 containing the K1303A mutation (lane 4), even though the wild-type and mutant proteins were produced at comparable levels (not shown). These results demonstrate that Mot1 is an essential component of ADI activity, and that the Mot1 polypeptide is likely to be sufficient for ADI activity.

We noted that a ternary complex composed of TBP, DNA, and the mutant Mot1(K1303A) protein was not detectable in these experiments (Fig. 3B, lane 4). Genetic data described below strongly suggest that overexpressed ATPase-defective Mot1 can interact with TBP in vivo, but this interaction is likely difficult to detect in vitro with the low levels of Mot1 produced in the in vitro translation reactions. It is also possible that Mot1(K1303A) does not interact with TBP–DNA complexes as strongly as wild-type Mot1, or that this mutant protein can still hydrolyze ATP but at a very slow rate.

Mot1 has ATPase activity

To determine whether Mot1 has ATPase activity, its 70-kD conserved carboxy-terminal domain, Mot1C (residues 1255–1867; Fig. 2), was expressed in *Escherichia coli* as a fusion to a (His)₆ tract to facilitate purification using nickel-chelate affinity chromatography. As a control, the same method was used to purify a mutant Mot1C protein containing a single amino acid substitution (D1408N). This residue lies within another sequence motif (DEGH) thought to be important for ATP binding and hydrolysis (Fig. 2) because substitution of the equivalent residue in eIF-4A has been reported to abolish ATPase activity, but not the ability to bind ATP (Pause and Sonenberg 1992). As an additional control, a purification was carried out in parallel with extract from cells expressing the vector alone. Equal amounts (140 ng) of Mot1C and Mot1C(D1408N), or an equal volume of the column eluate from the vector-only control, were

Figure 3. Mot1 possesses ADI activity. (A) Extracts from *mot1-1* cells do not contain detectable ADI activity. Nuclear extracts were prepared from wild-type *MOT1* and *mot1-1* mutant cells. These extracts were fractionated as described (Auble and Hahn 1993) and tested for ADI activity by a gel mobility retardation assay using a radiolabeled fragment of the adenovirus major late promoter and recombinant yeast TBP. Lane 1, incubation of extract from *MOT1* cells with TBP and DNA; lane 2, incubation of TBP, DNA, *MOT1* extract plus ATP; lane 3, incubation of TBP, DNA and extract from *mot1-1* cells; and, lane 4, incubation of TBP, DNA, *mot1-1* extract plus ATP. (B) In vitro translated Mot1 removes TBP from DNA. Activity of in vitro translated Mot1 was tested by the gel mobility retardation

assay. Incubation of TBP and DNA alone is shown in lane 1. Reactions in lanes 2–4 contain ATP which is present in the reticulocyte lysate. Lane 2, incubation of TBP, DNA and nonprogrammed reticulocyte lysate; lane 3, incubation of TBP, DNA and in vitro translated Mot1; and lane 4, incubation of TBP, DNA and in vitro translated Mot1(K1303A).

assayed for ATPase activity as described in Materials and methods. Neither the vector-only control nor the Mot1C(D1408N) mutant protein possessed any detectable ATPase activity above the buffer background; in contrast, the Mot1C protein displayed readily detectable ATPase activity (Fig. 4A). Unlike the Snf2 carboxy-terminal domain [Laurent et al. 1993], addition of single- or double-stranded DNA (with or without TATA sequences) did not significantly stimulate (≤ 2 -fold) the ATPase activity of Mot1C (data not shown). Under optimal conditions of pH and salt concentration, the specific activity of Mot1C was ~ 330 pmoles of ADP formed/ μ g per min, which is at least an order of magnitude higher than that reported for the Snf2 carboxy-terminal domain even upon its stimulation by double-stranded DNA [Laurent et al. 1993].

ATPase activity of Mot1 is required for its function in vivo

The D1408N mutation in the DEGH motif of Mot1C was inserted into the context of the full-length *MOT1* gene. To determine if this alteration was sufficient to compromise the function of Mot1 in vivo, the ability of the mutant gene to complement the slow growth (at 30°C) and temperature-sensitive (above 35°C) phenotypes of cells carrying a chromosomal *mot1-1* allele was examined. When expressed from its own promoter on a low-copy-number (centromere-containing) plasmid, the

wild-type *MOT1* gene was able to restore vigorous growth at 30°C and was able to complement the temperature-sensitive lethality of the *mot1-1* cells, unlike the vector alone (Fig. 4B, cf. upper left to upper right). In contrast, when the *MOT1*(D1408N) mutant gene was expressed from the same vector, it was unable to complement either the slow growth of the *mot1-1* cells at permissive temperature or their temperature-sensitive lethality (Fig. 4B, bottom left and right). When the centromere-containing plasmid carrying *MOT1*(D1408N) was introduced into *MATa*/*MATα* *mot1-Δ2::LEU2*/*MOT1* diploid cells and the resulting transformants subjected to meiosis, tetrad analysis demonstrated that the *MOT1*(D1408N) gene was also unable to complement the *mot1-Δ2::LEU2* null mutation (data not shown). Because both the wild-type *MOT1* gene and the *MOT1*(D1408N) mutant used in these experiments also carried a carboxy-terminal c-Myc epitope tag, it was possible to confirm by immunoblot analysis using anti-c-Myc monoclonal antibodies (Mab 9E10) that both the normal Mot1 and the Mot1(D1408N) mutant proteins were produced at essentially identical levels (data not shown). Thus, the D1408N substitution which abolished the ATPase activity of the protein in vitro (Fig. 4A) also eliminated its ability to function in vivo (Fig. 4B).

Most strikingly, however, both the *MOT1*(D1408N) mutation and the other presumptive ATPase-defective mutation, *MOT1*(K1303A), acted as strong dominant negative alleles. When these mutant genes were carried

Figure 4. Mot1 possesses an ATPase activity that is required for its function in vivo. (A) ATPase activity of the purified, bacterially-expressed carboxy-terminal domain of Mot1. The domain (Mot1C) of normal Mot1 (residues 1255-1867) that contains sequence motifs conserved in the Snf2/Swi2 family, known DNA and RNA helicases and other nucleic acid-dependent ATPases (Fig. 2), and a site-directed mutant (Mot1C D1408N) with an alteration in a putative ATP-binding element, were both expressed in *E. coli* and purified to near homogeneity as described in Materials and methods. The ATPase activity of samples of the eluate from the final step of purification (Ni^{2+} -chelate affinity chromatography) was measured by the assay method described in Materials and methods. No Enzyme, buffer only control; Vector, an equal volume of column eluate from a mock purification conducted with an extract from *E. coli* cells expressing empty vector; Mot1C, a sample (140 ng) of the normal carboxy-terminal domain; and, Mot1C D1408N, a sample (140 ng) of the mutant protein. Error bars represent the range of values observed for measurements made in duplicate. (B) A yeast strain (JDY215b) containing the *mot1-1* temperature-sensitive allele [Davis et al. 1992] was transformed with either a centromere-containing vector (pRS316) alone (upper right quadrant), or the same vector expressing the normal *MOT1* gene from its own promoter (upper left quadrant), or the same vector expressing the *mot1*(D1408N) mutant from its own promoter (lower left and right quadrants). Transformants harboring these plasmids were streaked to single colonies on SCD-Ura plates and incubated at temperatures both permissive (30°C) and restrictive (35°C) for the *mot1-1* host cell.

on multi-copy (2 μ m DNA-containing) plasmids and were overexpressed from the galactose-inducible *GAL1* promoter in *MOT1*⁺ cells, growth was dramatically inhibited (Fig. 5A, lower right and left). In contrast, the presence of the vector alone or overexpression of normal *MOT1* had little or no effect on the growth of wild-type cells (Fig. 5A, upper left and right). These results suggested the possibility that without ATP hydrolysis, the mutant Mot1 may become locked in a nonfunctional complex with TBP.

Genetic interactions between *MOT1* and TBP

Because the biochemical findings presented here demonstrated that Mot1 is ADI, and because it was shown previously that ADI interacts stereospecifically with TBP in vitro, we tested whether evidence for functional association of Mot1 and TBP in vivo could be obtained through genetic interactions between *MOT1* and TBP.

Indeed, when TBP (encoded by the *SPT15* gene) was constitutively over-expressed from the *ADH1* promoter on a multi-copy (2 μ m DNA-containing) plasmid, it had

no effect on the growth of wild-type cells, regardless of whether they were overproducing normal Mot1 (Fig. 5B, upper half). Strikingly, however, overexpression of TBP rather efficiently suppressed the lethality caused by overproduction of the dominant-negative Mot1-(D1408N) mutant (Fig. 5B, lower half). Likewise, overexpression of TFIIA (encoded by the *TOA1* and *TOA2* genes) from a 2 μ m DNA-containing plasmid also suppressed the growth inhibitory effect of the dominant-negative Mot1(D1408N) mutant, but significantly less well than TBP (Fig. 5C). Overexpression of TFIIIB (encoded by the *SUA7* gene), also carried by a 2 μ m DNA-containing plasmid, was unable to suppress the dominant negative effect of the *MOT1* (D1408N) allele (data not shown).

In contrast to the ability of overproduced TBP to reverse the effect of the dominant-negative *MOT1*-(D1408N) allele, overproduction of TBP dramatically exacerbated the slow growth phenotype of cells carrying the recessive *mot1-1* mutation. In fact, *mot1-1* cells transformed with the plasmid over-producing TBP grew extremely slowly and were difficult to maintain because

Figure 5. TBP overproduction overcomes the growth inhibitory effect of a dominant negative *MOT1* allele and exacerbates the phenotype of the *mot1-1* mutation. (A) A *MOT1*⁺ strain, W303-1A (WT), was transformed with either a multi-copy *GAL1* promoter-containing vector (YE352G) alone (upper left quadrant), or the same vector expressing from the *GAL1* promoter either the normal *MOT1* gene (upper right quadrant) or two different site-directed *mot1* mutants, each containing an alteration in a sequence motif thought to be involved in ATP binding and hydrolysis, *MOT1*(K1303A) (lower left quadrant) and *MOT1*(D1408N) (lower right quadrant). Transformants harboring these plasmids were streaked to single colonies and incubated at 30°C either on SCD-Ura plates (Glc) for 2 days or on SCGalSuc-Ura plates (Gal) for 4 days. (B) Strain W303-1A transformed with YE352G expressing either normal *MOT1* (upper half) or the dominant negative allele, *MOT1*(D1408N) (lower half), were cotransformed with either a multi-copy *ADH1* promoter-containing vector (AAH5) alone (left side) or the same vector expressing yeast TBP (*SPT15* gene product) from the *ADH1* promoter (right side). The transformants were then grown as in A (except that the media also lacked Leu). (C) Strain W303-1A transformed with YE352G expressing either normal *MOT1* (upper half) or the dominant negative allele, *MOT1*(D1408N) (lower half), were cotransformed with either a multi-copy vector (YE351) alone (left side) or the same vector expressing both of the subunits of yeast TFIIA (*TOA1* and *TOA2* gene products) from their own promoters (right side). The transformants were then grown as in B. (D) Either a *mot1-1* mutant, JDY215b (right side), or its otherwise isogenic *MOT1*⁺ parent, JDY194 (WT; left side), each harboring a plasmid (YE352G) expressing *MOT1* under control of the *GAL1* promoter, were transformed with either a multi-copy *ADH1* promoter-containing vector (AAH5) alone (upper half) or the same vector expressing yeast TBP from the *ADH1* promoter (lower half). The transformants were selected initially on galactose-containing medium (SCGalSuc-Ura-Leu plates), and then individual isolates were streaked to single colonies on either galac-

tose-containing medium or glucose-containing medium (SCD-Ura-Leu plates) to repress expression of the plasmid-borne *MOT1* gene and incubated for 4 days at a temperature (30°C) otherwise permissive for the growth of the *mot1-1* host cell.

of the strong selective pressure for reversion of the *mot1-1* mutation or loss of the TBP-expressing plasmid (data not shown).

To avoid this problem, and to permit a more direct test of the effect of overproduced TBP in *mot1-1* mutants, both *mot1-1* cells and otherwise isogenic *MOT1*⁺ cells were first transformed with a plasmid expressing normal *MOT1* from the *GAL1* promoter and selected on galactose-containing plates. These transformants were then cotransformed on galactose-containing medium with the plasmid constitutively overexpressing TBP under control of the *ADH1* promoter or the corresponding vector. To determine the effect of TBP overexpression, the cells were then streaked to single colonies on glucose-containing plates to repress transcription of the plasmid-borne copy of normal *MOT1*. As anticipated, in the absence of normal Mot1 expression, overproduction of TBP severely inhibited the growth of *mot1-1* mutants (Fig. 5D, right side), whereas TBP overexpression had no effect on the growth of the *MOT1*⁺ cells (Fig. 5D, left side).

MOT1 represses pol II but not pol I and pol III transcription in vitro

TBP is required for transcription by all three nuclear RNA polymerases. Because Mot1 is targeted to TBP–DNA complexes, it was possible that Mot1 affects the expression of genes transcribed by RNA polymerases I and/or III, as well as those transcribed by pol II. To test the specificity of Mot1, nuclear extracts were prepared from *MOT1*⁺ and *mot1-1* cells, and these were used for in vitro transcription experiments. As assayed by primer extension, basal pol II transcription was elevated between 5- and 15-fold when transcript levels produced in extracts from *mot1-1* cells were compared to those produced in extracts from wild-type cells (Fig. 6A). This effect was not promoter-specific because basal expression from each of three different pol II promoters was affected similarly. In contrast, levels of activated transcription stimulated by Gal4–VP16 were similar in *MOT1* and *mot1-1* extracts (Fig. 6B).

Similar results were obtained in vivo using reporter plasmids in which yeast promoters were used to drive the expression of *E. coli* β -galactosidase. It had been shown previously using hybridization analysis to blots of poly(A)⁺ RNA that basal expression, but not activated transcription, of a number of genes is markedly elevated in the *mot1-1* mutant (Davis et al. 1992). To address what promoter elements might be responsible for mediating the repressive effect of Mot1, matched sets of four different promoters, either containing or lacking their known upstream activation sequences (UAS), were fused to *lacZ* and introduced on multi-copy plasmids into *MOT1*⁺ and *mot1-1* cells. In all cases, removal of the UAS elements greatly lowered the level of transcription, as expected (Table 1). However, for three of the four promoters examined (the glucose-regulated *CYC1* promoter, the phosphate-regulated *PHO5* promoter, and the relatively constitutive *MFa2* promoter), expression from the TATA-containing basal elements of these UAS-less con-

Figure 6. Mot1 is an RNA polymerase II-specific transcription factor. In vitro transcription experiments were performed using nuclear extracts obtained from wild-type *MOT1* or *mot1-1* mutant cells, as indicated. (A) Basal RNA polymerase II transcription from the *CYC1*, *ACT1*, and *HIS4* promoters is shown. Correctly initiated transcripts detected by primer extension are bracketed. (B) RNA polymerase II transcription from the *CYC1*, *ACT1*, and *HIS4* promoters activated by Gal4–VP16 is shown. Correctly initiated transcripts are bracketed. (C) Transcription of sup4 tRNA, 5S, U6 and 35S templates is shown. Pol I transcripts were detected by S1 nuclease protection and pol III was assayed by run-off transcription.

structs was dramatically affected in the *mot1-1* cells, increasing 87-fold, 4-fold, and 15-fold, respectively (Table 1). These differences are not attributable to plasmid copy number because the plasmid copy number differs less than twofold between *MOT1* and *mot1-1* cells (Davis et al. 1992). In contrast, when basal expression was elevated by the presence of a UAS, the change in transcription in *mot1-1* cells was small. The pheromone-inducible *FUS1* promoter (with or without its UAS) was found to be nonresponsive to the *mot1-1* mutation. In the absence of a UAS, β -galactosidase levels driven by the *FUS1* promoter were nearly undetectable. Because the absence of fully functional Mot1 significantly increased gene expression driven by the basal elements of several different promoters that are normally under diverse types of regulatory controls, these observations are consistent with a role for normal Mot1 in repressing expression at TATA sequences rather than elsewhere within these promoters.

In contrast to the effects of the *mot1-1* mutation on

Table 1. Expression of promoter-lacZ fusions in *MOT1* and *mot1-1* cells

Carbon source ^a	Vector	Promoter	β -Galactosidase activity ^b		Change fold (+ or -)
			<i>MOT1</i>	<i>mot1-1</i>	
Glycerol-lactate	pLGA-312S	<i>CYC1</i>	3356 $\pm$ 102	no growth	—
Glycerol-lactate	pLGA-178	Δ UAS- <i>CYC1</i>	1.3 $\pm$ 0.45	no growth	—
Glucose	pLGA-312S	<i>CYC1</i>	956 $\pm$ 102	4141 $\pm$ 873	4 (+)
Glucose	pLGA-178	Δ UAS- <i>CYC1</i>	0.59 $\pm$ 0.14	52 $\pm$ 9.4	87 (+)
Glucose	pMH313	<i>PHO5</i>	50 $\pm$ 22	57 $\pm$ 8.2	0
Glucose	pMH324	Δ UAS- <i>PHO5</i>	12 $\pm$ 4.3	50 $\pm$ 10	4 (+)
Glucose	pCM100	<i>MFa2</i>	3600 $\pm$ 524	2114 $\pm$ 208	2 (-)
Glucose	pCM100.1	Δ UAS- <i>MFa2</i>	3.0 $\pm$ 1.3	44 $\pm$ 9.5	15 (+)
Glucose	pCM101	<i>FUS1</i>	4.1 $\pm$ 0.73	7 $\pm$ 1.5	0
Glucose	pCM101.1	Δ UAS- <i>FUS1</i>	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0	0
Glucose	—	—	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0

^aCells were grown in minimal medium containing 2% glucose or in minimal medium containing 3% glycerol and 2% DL-lactate.

^bValues given represent the averages ($\pm$ S.D. of the mean) for at least two or three independent measurements of specific activity, each conducted in triplicate. Specific activity is expressed as nmoles of ONPG cleaved per min/mg of protein at 28°C; 1 nmole of o-nitrophenol gives an A_{420} = 0.0045 (Miller 1972).

pol II transcription, transcription of a pol I gene (35S rRNA) and two pol III genes (tRNA and 5S rRNA) was enhanced only slightly ($\leq$ 2-fold) when extracts from *mot1-1* cells were compared to extracts from *MOT1*⁺ cells (Fig. 6C). It is possible that *MOT1* has only a weak effect on pol I and pol III genes because they are controlled by very strong promoters. The yeast U6 gene was weakly but detectably transcribed by RNA polymerase III in these extracts and the levels of transcription were the same in extracts from *MOT1* and *mot1-1* cells. Although the U6 gene is transcribed by RNA polymerase III, it contains a functionally important TATA box which binds TBP (Eschenlauer et al. 1993; Joazeiro et al. 1994). Taken together, these results suggest that Mot1 is an RNA polymerase II-specific transcriptional repressor and the presence of a TATA box is not necessarily sufficient to confer regulation by Mot1.

Discussion

Mot1 represents a novel type of transcriptional repressor

Studies on repression of transcription independently applying biochemical and genetic methods have led to identification of the same factor. ADI was originally detected as an ATP-dependent activity that can remove TBP bound to DNA and that represses transcription in vitro. *MOT1* was identified by a mutation which caused derepression of the transcription of many genes in yeast. Mot1 is essential for cell viability and its sequence contains consensus motifs involved in ATP binding and hydrolysis.

The evidence presented here strongly suggests that ADI and Mot1 are identical and these results provide insight about the molecular mechanism of action of Mot1. First, extracts from cells containing a temperature-sensitive allele (*mot1-1*) do not contain detectable ADI activity. Second, loss of ADI activity is correlated

with increased basal transcription of genes transcribed by pol II both in vivo and in vitro. Third, like the specificity observed for association of ADI and TBP in vitro (Auble and Hahn 1993), *MOT1* and TBP interact genetically in vivo, with TBP overexpression enhancing the phenotype of the recessive *mot1-1* mutation and suppressing the phenotype of the dominant *MOT1(D1408N)* and *MOT1(K1303A)* mutations. Fourth, Mot1 contains an ATPase activity which is essential for its function both in vivo and in vitro. Finally, in vitro translated Mot1 can dissociate TBP from DNA in an ATP-dependent manner, just like ADI. It is formally possible that another required component of ADI is present in the reticulocyte lysate but we think this is unlikely.

Mot1 functions to repress basal and weakly activated transcription, but has little or no effect on highly transcribed genes. For example, transcription in vivo of the strongly expressed calmodulin promoter (Davis et al. 1992) and in vitro transcription stimulated by Gal4-VP16 (Fig. 6) are not significantly elevated in the absence of *MOT1* function. Likewise, basal transcription in vitro can be repressed by histones, and this chromatin-mediated repression can be overcome by the presence of transcriptional activators (Durrin et al. 1991; Laybourn and Kadonaga 1992; Workman and Kingston 1992). Mammalian repressors of transcription identified biochemically, such as NC1, NC2, and topoisomerase I, also resemble Mot1 in that these factors selectively repress basal but not activated transcription (Meisterernst and Roeder 1991; Meisterernst et al. 1991; Kretzschmar et al. 1993; Merino et al. 1993). However, despite this superficial similarity, the mammalian repressors characterized to date and histone-mediated repression are likely to act by mechanisms that are distinct from Mot1 because histones and these mammalian factors have no known homology to Mot1 and do not appear to require ATP for their function. Thus, we propose that Mot1 represents a paradigm for a previously unknown type of transcriptional repressor whose function is to keep basal tran-

scription low by interacting directly with TBP bound to DNA and catalyzing its removal at the expense of ATP hydrolysis.

The essential function of Mot1

Cells carrying *mot1Δ* null alleles are inviable. Cells carrying the *mot1-1* temperature-sensitive allele grow slowly even at the permissive temperature. Why is MOT1 function essential for growth and viability?

The *in vitro* studies presented here corroborate the conclusion from previous genetic analysis that *mot1-1* cells are deficient in Mot1 activity *in vivo*. The fact that *mot1-1* cells cannot tolerate an elevated level of TBP (Fig. 5D) suggests that one essential role for Mot1 action may be to prevent the indiscriminate binding of TBP to inactive promoters, thus preventing inappropriate gene expression. Conversely, the ATPase-defective MOT1 alleles are dominant negative apparently because they sequester TBP and/or promote promiscuous binding of TBP to inactive promoters, thus preventing inappropriate gene expression. Conversely, the ATPase-defective MOT1 alleles are dominant negative apparently because they sequester TBP and/or promote promiscuous binding of TBP to inactive promoters, thus preventing inappropriate gene expression. Conversely, the ATPase-defective MOT1 alleles are dominant negative apparently because they sequester TBP and/or promote promiscuous binding of TBP to inactive promoters, thus preventing inappropriate gene expression.

Models for Mot1 function in transcriptional regulation

Because the binding of TFIID to a promoter is the first step in the formation of a transcription initiation complex, it is a likely point for gene regulation. TFIIA can block the action of Mot1 *in vitro* (Auble and Hahn 1993), and TFIIA also partially suppresses the growth defect in cells harboring dominant-negative MOT1 alleles (Fig. 5C). Mot1-mediated transcriptional repression can also be overcome by transcriptional activators, like Gal4-VP16. One of the functions of site-specific activators might be to recruit TFIIA as a means to block Mot1 action. On the other hand, transcriptional activators or other general factors may interact directly with TBP (or via other proteins) to block the action of Mot1. Poon et al. showed by immune precipitation that Mot1 is one of several TBP associated factors (TAFs) (Poon and Weil 1993; D. Poon, A.M. Campbell and P.A. Weil, pers. comm.) Because Mot1 has a specific effect on pol II transcription *in vitro*, we presume that it is a pol II-specific TAF.

One model for the role of Mot1 in repressing gene expression is that it makes the binding of TFIID to DNA rate-limiting by increasing the dissociation rate of TBP from the TATA element (Fig. 7A). *In vitro*, TBP-TATA complexes are quite stable (Hahn et al. 1989; Lieberman et al. 1991). Presumably, in the absence of Mot1, the dissociation of TFIID from DNA would also be slow. In

Figure 7. Models for Mot1 function *in vivo*. (A) TBP binds to the TATA box at promoters transcribed by RNA polymerase II (center). Mot1 removes TBP from DNA in an ATP-dependent manner (left). If TFIIA binds to TBP first, however, Mot1 is prevented from functioning (right). Mot1 action is also blocked by strong transcriptional activators. *In vivo*, TBP is an essential component of TFIID, a large multi-subunit complex. (B) Mot1 may remove TBP from the promoter in an ATP-dependent manner once RNA polymerase II transcription has begun. (C) An additional, but not exclusive, role for Mot1 may be to assist in correctly orienting TBP at symmetrical TATA boxes by catalyzing dissociation of TBP from the promoter in an ATP-dependent manner, thus allowing TBP to rebind in a mode productive for transcription initiation.

the presence of Mot1, however, TFIID binding to promoters *in vivo* would be unstable unless TFIID binding were stabilized by interaction with another factor (such as TFIIA, other basal factors, or a transcriptional activator) that is able to block Mot1 action (Fig. 7A). This model could explain how Mot1 selectively represses basal transcription. Testing of this model awaits purification of yeast TFIID complexes that contain Mot1.

Another possible role of Mot1 in regulating gene expression is to accelerate dissociation of TFIID from DNA once the elongating polymerase has disengaged from the preinitiation complex (Fig. 7B). *In vitro* studies with highly purified and/or recombinant basal transcription factors demonstrate that TBP remains at the promoter after pol II moves along the template (D. Reinberg, pers. comm.). In contrast, *in vitro* studies in crude extracts suggest that TFIID, as well as other factors, dissociate as pol II elongates a chain (Kadonaga 1990). Mot1-mediated dissociation of TFIID after transcription initiation has occurred would allow the rebinding of TFIID to be a regulated step for subsequent rounds of transcription initiation.

A related function for Mot1 may involve facilitating the correct polarity of TBP binding to DNA (Fig. 7C). Many TATA sequences are symmetrical, but TBP is asymmetric. The orientation of TBP binding with respect to the promoter is critical for proper gene expression (Nagawa and Fink 1985). TBP bound in the incorrect orientation at a symmetrical TATA would interfere with transcription because TBP–DNA complexes dissociate slowly. Mot1 could, however, accelerate dissociation of incorrectly positioned TBP and thereby provide another opportunity for TBP binding in the correct orientation (Fig. 7C). Further genetic and biochemical studies should allow testing of these various models for Mot1 function.

In view of the evolutionary conservation of the pol II transcription machinery, and the fact that Mot1 belongs to a highly conserved family, it would not be surprising if Mot1 itself has been conserved. Although no Mot1 homolog has yet been identified as a pol II TAF in humans or flies, it may be more weakly associated with the TFIID complex than other TAFs. In fact, Mot1 is readily dissociated from yeast TBP by ion exchange chromatography (Auble and Hahn 1993).

Function of Snf2/Swi2 family members

Mot1 is a member of the family of Snf2/Swi2-related regulatory factors. These factors all contain a large (~550 residue) conserved domain, but are largely divergent outside of this conserved region (Fig. 2). Proteins containing this highly conserved domain are found in organisms as diverse as bacteria and humans. Genetic and biochemical data suggest that Snf2/Swi2 is part of a large complex required for expression of many yeast genes and that it acts by overcoming repression by nucleosomes (Happel et al. 1991; Hirschhorn et al. 1992; Laurent and Carlson 1992; Peterson and Herskowitz 1992; Winston and Carlson 1992; Cairns et al. 1994). *Drosophila brm* is required for activation of a set of homeotic genes (Tamkun et al. 1992). ERCC6/CSBC, Rad54, Rad16, and Rad5 are involved in repair of DNA damage (Bang et al. 1992; Johnson et al. 1992; Toelstra et al. 1992; Emery et al. 1993). *Drosophila lodestar* is required for proper segregation of chromosomes (Girdham and Glover 1991). Finally, Mot1 is a repressor of basal transcription.

How can these highly related proteins have such diverse roles in cellular regulation? We speculate that the conserved region encodes a catalytic domain that uses ATP for a conserved enzymatic function. Consistent with this view, swapping of the conserved domains from two other family members for the corresponding segment in Snf2 restored partial Snf2 function (Khavari et al. 1993; Laurent et al. 1993). By analogy with the mechanism we have described here for Mot1, we propose that the conserved domain in each of the Snf2/Swi2 family members functions to remove proteins from DNA at the expense of ATP hydrolysis. ATPase activity is essential for the functions of Mot1 as well as Snf2/Swi2 and Brg1, a human Snf2/Swi2 family member (Khavari et al. 1993; Laurent et al. 1993). We propose that the different proteins have different functions because they are targeted

to different protein–DNA complexes. The nonconserved regions of these molecules could be responsible for the unique protein–protein interactions that allow them to dissociate different protein–DNA complexes in an ATP-dependent manner for different purposes. For example, genetic and biochemical data suggest that Snf2 alters chromatin structure (Winston and Carlson 1992); the possible ATP-dependent removal of histones from DNA by the Snf2 complex is a function entirely analogous to the ATP-dependent removal of TBP from DNA that is carried out by Mot1. Just as Mot1 appears to be a component of the TFIID complex, genetic and biochemical data also suggest that Snf2 functions in a large complex with other proteins important for transcriptional activation (Laurent and Carlson 1992; Cairns et al. 1994). Similarly, ERCC6/CSBC and the Rad proteins could be targeted to repair enzymes bound to DNA at sites of DNA damage where the removal of proteins may be an obligate part of the repair process. Thus, the nonconserved regions of Snf2/Swi2 family members may mediate their interactions with other proteins that determine the target specificity of their action. It is also possible that the target specificity of Snf2/Swi2 family members overlaps with the conserved region as the replacement of the Snf2/Swi2 conserved region with the conserved region from the related *Drosophila ISWI* gene does not restore Snf2/Swi2 function (Elfring et al. 1994).

It was proposed initially that all members of the extended Snf2/Swi2 family might be ATP-dependent helicases (Davis et al. 1992; Laurent et al. 1992; Bork and Koonin 1993). However, a more recent computer analysis suggests that Mot1 and the other Snf2/Swi2 family members are distinct from helicases because they share their highest degree of sequence similarity with viral factors that appear to lack helicase activity in vitro (Henikoff 1993). Moreover, the Snf2 carboxy-terminal domain (Laurent et al. 1993) and purified ADI (Auble and Hahn 1993) apparently do not have detectable helicase activity in vitro. However, it is possible that ATP hydrolysis by Mot1, Snf2, and the other related factors stimulates dissociation of protein–DNA complexes by inducing structural alterations in DNA, such as local transient unwinding.

Apart from its ability to hydrolyze ATP, Mot1 is the first Snf2/Swi2 family member whose biochemical activity—dissociation of TBP from TATA-containing DNA—has been elucidated. Further studies are required to determine how Mot1 catalyzes removal of TBP from DNA and whether this mechanism of protein–DNA dissociation is conserved among the other family members.

Materials and methods

Strains and growth conditions

Yeast strains used in this study were W303-1A (MATa MOT1⁺ *ura3-1 his3-11,15 ade2-1 leu2-3,112 trp1-1 can1-100*), JDY194 (MATa MOT1⁺ *ura3-52 his4-519 leu2-3,112 trp1-1 can1-101*) and JDY215b (*mot1-1*, otherwise isogenic to JDY194) (Davis et al. 1992). Yeast cells were propagated and DNA-mediated transformation and other genetic manipulations were performed as

recommended (Sherman et al. 1986). GalSuc media contained 2% galactose and 0.2% sucrose as the carbon sources; glucose media contained this carbon source at 2%.

Purification of ADI

All buffers contained protease inhibitors as described previously (Auble and Hahn 1993) and fractionation was performed using siliconized glassware whenever possible. ADI was purified from yeast nuclear extract through the BioRex 70 step essentially as described (Auble and Hahn 1993). Fractions containing the peak of ADI activity were pooled and the conductivity was adjusted to that of HA buffer (30 mM HEPES at pH 8, 2 mM EDTA, 10% glycerol, 0.1% Brij 58, 1 mM DTT) plus 0.1 M potassium acetate by dialysis and dilution. On average, ~0.6 mg of protein was obtained from 450 g wet weight of late-exponential phase yeast cells. This material was loaded onto a 0.5 ml Macroprep CM column and eluted with an 8.5 ml gradient of potassium acetate from 0.1–0.7 M in HA. ADI eluted at ~0.3 M salt. To obtain enough ADI polypeptide for amino acid sequence analysis (see below), ADI activity from three separate preparations (119,000 units of activity, 1.86 mg) was pooled and the protein was precipitated with TCA. The pooled protein was then fractionated on a Superose-12 column in 6 M guanidine HCl, 50 mM Tris-HCl (pH 8), 0.1% Brij 58 and 1 mM DTT. The fractions containing the ADI polypeptide were identified by SDS-PAGE followed by silver-staining. The Superose-12 fractions containing the ADI polypeptide were pooled and dialyzed against 30 mM Tris-acetate (pH 8), 2 mM EDTA, 10% glycerol, 25 mM potassium acetate, 0.1% Brij 58, 1 mM DTT. The dialyzed protein was then precipitated with TCA and fractionated on a 5% polyacrylamide gel. The ADI material was transferred to a PVDF membrane (Bio-Rad) in Towbin transfer buffer (Towbin et al. 1979) (without SDS). The ADI polypeptide was localized by staining the PVDF membrane with 0.1% Ponceau S as described (Ranish et al. 1992) and excised for further analysis.

Trypsin digestion, HPLC separation and protein sequencing

PVDF slices containing ADI were submitted to in situ digestion with trypsin (Fernandez et al. 1992). The resulting peptide mixture was separated by narrow-bore high performance liquid chromatography using a Vydac C18 reverse-phase column (2.1 mm x 150 mm) on a Hewlett-Packard 1090 HPLC with a 1040 diode array detector. Optimum fractions were chosen based on symmetry, resolution, ultraviolet absorbance spectra, and submitted to automated Edman degradation on an Applied Biosystems 477A protein sequencer. Strategies for peptide selection, reverse-phase separation and protein sequencing have been described previously (Lane et al. 1991).

Photocross-linking of ADI to DNA

Two DNA probes were synthesized. The 41-mer 5'-GATC-CCCGGTGTAGACAGAAGAGGGCTATAAAAGGGGGTG-3' was annealed to either 5'-CCACCCCTTTTATAGCCC-3' (probe 1) or 5'-CCACCCCTTTTATAGCCCTCTT-3' (probe 2). Partial fill-in reactions were then performed as described (Bartholomew et al. 1991) in near darkness using exonuclease-free Klenow fragment of DNA polymerase and 20 μ M photocrosslinking nucleotide, 5-[N-(p-azidobenzoyl)-3-aminoallyl]dUMP (kindly provided by G.A. Kassavetis and E.P. Geiduschek) with either [α -³²P]dCTP (probe 1) or [α -³²P]dCTP and 0.1 mM dGTP (probe 2). Following a 15 min incubation at 37°C, fully duplex DNA was synthesized by adding 0.5 mM dGTP, dTTP, dATP, and dCTP to each reaction. Following a second 15

min incubation at 37°C, the DNAs were ethanol precipitated and purified on a nondenaturing-polyacrylamide gel. Binding reactions were performed using the photocrosslinking probes exactly as for ordinary radiolabeled probes except that the binding reactions contained 5 mM β -mercaptoethanol instead of DTT. Following incubation at room temperature for 20 min, samples were irradiated using a handheld short-wave UV light (UVGL-25 mineralight, UVP Inc.) 1.5 cm above the tops of the open Eppendorf tubes which contained the binding reactions. Half of each reaction was then transferred to a new tube to which was added DTT to 20 mM (to terminate the cross-linking reaction), and the quenched reaction mixtures were loaded on a mobility retardation gel as described previously (Auble and Hahn 1993). To the remaining half of the reaction mixtures was added CaCl₂ to 5 mM, 1 μ g of DNase I and 19 units of micrococcal nuclease. The nuclease-treated reactions were incubated for 15 min at 37°C, then mixed with an equal volume of SDS sample buffer, boiled and loaded on 6 or 10% SDS-polyacrylamide gels. The crosslinked products were detected by autoradiography. No crosslinking was detected in the absence of UV light, no crosslinking to TBP alone was detected, and the amount of crosslinked protein was proportional to the dose of UV light for up to two min of irradiation (not shown).

Site-directed mutagenesis

pKH10, which encodes residues 1255–1867 (613 amino acids) of Mot1 plus an in-frame c-Myc epitope tag (LEEQKLISEEDLLRKR) at its carboxyl terminus, was constructed by inserting the *Bam*HI fragment of pRS7.1myc (K. Hansen, J. Davis, and J. Thorne, in prep.) into M13mp18. Single-stranded DNA produced from this plasmid was mutagenized using a commercial oligonucleotide-directed in vitro mutagenesis system (Amersham, Version 2.1). The mutagenic oligonucleotide primer, 5'-GTGTTTGAATGAAGGAC-3', was used to introduce a single base change that converted the Asp codon at residue 1408 of Mot1 to an Asn codon. The complete nucleotide sequence of the 1.2-kb *Hpa*I–*Eco*NI segment of the resulting plasmid, pKH11, was determined to confirm the presence of the mutation and the absence of extraneous mutations. This fragment was substituted for the corresponding region of *MOT1* in pRS7.1myc, to create pKH12.

In an essentially identical manner, another mutagenic oligonucleotide primer, 5'-GATATGGGTTTGGGTGCTACATTACAAACCATC-3', was used to introduce a single base change that converted the Lys codon at residue 1303 of Mot1 to an Ala codon. This mutation was verified by sequence analysis and introduced, as a control, into the *MOT1* template used for production of Mot1 by in vitro transcription and translation (see below).

Plasmid constructions

The ends of the *MOT1* coding sequence were adapted for in vitro expression by performing PCR using 5'-ACTTGTTG-GATCCTGGCCACGTCACGAGTTTCGAGGCTG-3' and 5'-CCAAGAAGGAGCATGGGCAACTAT-3' for amplification of the 5' end and 5'-CTGAGCGCATTGAGAACATTACTC-3' and 5'-CTGACTTGTTCGGCCGCGAGATCTTTATCGTAAAGTTTTGATGAAGGT-3' for amplification of the 3'-end using plasmid pMR13 (Piatti et al. 1992) as the template. Restriction digestion of these products generated a *Bam*HI–*Pst*I 5'-end fragment and an *Eco*RV–*Not*I 3'-end fragment. Each of these was subcloned into pBluescript II SK(+) and verified by nucleotide sequencing. The 5'-end also contained a *Msc*I site suitable for introduction into a T7 expression vector in which the *MOT1*

coding sequence was fused to the CITE translational enhancer (Ranish et al. 1992). The full-length *MOT1* sequence or the *MOT1(K1303A)* mutant were introduced into this T7 vector by subcloning the engineered 5' end, followed by the insertion of the remaining portion of the gene with the *NotI* 3'-end.

The carboxyl terminus of Mot1, which includes the domain of homology to Snf2/Swi2, was expressed from the bacteriophage T7 promoter in *E. coli* as a fusion to a tract of six histidines by inserting the 1.8-kb *BamHI* fragment of pRS7.1myc into pET15b (Novagen), to yield pKH9. This plasmid produces a protein, Mot1C, whose amino-terminal sequence is MGSSHH-HHHHSSGLVPRGSHMLE followed by the carboxy-terminal 613 amino acids of Mot1 and the c-Myc epitope tag. pKH13 is otherwise identical to pKH9, except it contains the D1408N mutation, and it was constructed by inserting the 1.8-kb *BamHI* fragment of pKH12 into pET15b.

To express *MOT1* from the *GAL1* promoter, PCR was used to introduce a *XhoI* site just 5' to the ATG of the *MOT1* coding sequence. This amplification was accomplished using a mutagenic 5' primer (5'-CCCTCGAGCAAATGACGTCACG-3') and a nonmutagenic 3' primer (5'-CCATTGATCGGCAGCC-3') which annealed from +61 to +77. The resulting 88-bp PCR product was digested with *XhoI* and *BspEI* and used to replace the 3-kb *XhoI*-*BspEI* fragment of pRS7.1BgIII [which consists of a *MOT1*-containing *BglII* fragment inserted into pRS316 (Davis et al. 1992)], to create pKH2. pKH6 was constructed by inserting the *MOT1*-containing *XhoI*-*NaeI* fragment of pKH2 into YEp352Gal (Benton et al. 1990), which was digested with *HindIII*, blunt-ended by incubation with T4 DNA polymerase, and then cleaved with *SalI*. YEp352Gal contains the *GAL1-10* promoter, a 2 μ m origin of replication, and a *URA3* marker. To attach the carboxy-terminal c-Myc epitope tag, the 2.9-kb *SacI* fragment from pKH6 was replaced with the 2.4-kb c-Myc tag-containing *SacI* fragment from pRS7.1myc, to produce pKH7, which overexpresses epitope-tagged *MOT1* from the *GAL1* promoter. pKH14, which overexpresses c-Myc-tagged *MOT1* (D1408N), was created in an essentially identical fashion by swapping the 2.9-kb *SacI* fragment of pKH6 with the 2.4-kb *SacI* fragment from pKH12. pKH20 is identical to pKH7 and pKH14, but contains the *MOT1(K1303A)* mutation.

A LEU2- and 2 μ m DNA-containing plasmid (pAAH5) expressing yeast TBP (*SPT15* gene product) from the *ADH1* promoter (Gill and Tjian 1991) was the gift of Dr. Grace Gill. A *LEU2*- and 2 μ m DNA-containing plasmid (pSH346) expresses both subunits of yeast TFIIA (*TOA1* and *TOA2* gene products) from their endogenous promoters (S. Hahn, unpublished results).

pLGA178 (Guarente and Mason 1983) and pLGA312S (obtained from Dr. Michael Botchan) contain the *CYC1* promoter from +4 to -178 and from +4 to -312, respectively, fused to the *E. coli lacZ* gene. The *CYC1* promoter in pLGA178 lacks UAS1 and UAS2 (Guarente et al. 1984). pLGA312S is identical to pLGA312 (Guarente and Mason 1983), except for the presence of a *SalI* linker inserted at position -178 (*XhoI*). pCM100 and pCM100.1 were derived from pSM38 (a gift from Dr. Susan Michaelis), which contains the *MFa2* promoter and 32 amino acids of the coding sequence fused to the *E. coli lacZ* gene. To generate pCM100, a *HindIII* (blunt-ended by incubation with T4 DNA polymerase) and *StuI* fragment from pSM38, which contains the *MFa2-lacZ* fusion from -675 (numbering from the start site of translation) was ligated into the *XhoI* (which was converted to flush ends) and *StuI* sites of pLGA178T (Davis et al. 1992), in place of the entire *CYC1* cassette. pCM100.1 was generated in a similar way, except that the *MFa2* promoter was truncated to position -191 at the *NsiI* site (again blunt-ended) before insertion into pLGA178T. This deletion removes the

Mcm1 binding site and the three most distal Ste12 binding sites (Dolan et al. 1989). pCM101 and pCM101.1 were derived from pSB234 (a gift from Dr. Joshua Trueheart), which contains the entire *FUS1* promoter and 256 amino acids of coding sequence fused to the *E. coli lacZ* gene. To generate pCM101, a *SnaBI*-*StuI* fragment of the *FUS1-lacZ* fusion from -402 (numbering from the start site of translation) was inserted into the *XhoI* (blunted) and *StuI* sites of pLGA178T. pCM101.1 was constructed in a similar way, except that the *FUS1* promoter was truncated to position -124 at the *DraI* site prior to insertion into pLGA178T. This deletion removes all of the sequences required for regulated expression of the *FUS1* gene (Hagen et al. 1991). pMH313 and pMH324 (both gifts of Dr. Michael Grunstein) contain, respectively, the entire promoter and upstream region of the *PHO5* gene and the region of the *PHO5* promoter containing only its TATA-element (Han and Grunstein 1988).

Assay of β -galactosidase activity

Enzyme assays were performed, in duplicate or triplicate, as described previously (Rose et al. 1981; Hagen and Sprague 1984). Briefly, yeast cells were grown in minimal medium under selective pressure to an A_{600} nm of 1–2, harvested, and whole-cell extracts were prepared by glass bead lysis in the presence of PMSF (0.1 mg/ml). β -Galactosidase activity in the extracts was measured as described (Miller 1972) and normalized to the protein concentration of the whole-cell extract. By this procedure, even very low levels of β -galactosidase could be accurately measured.

Analysis of *MOT1* activity

Mot1 was made in vitro using a coupled in vitro transcription/translation system as described by the manufacturer (Promega). Mot1 activity in the in vitro translation products was assayed by gel mobility retardation experiments as described previously (Auble and Hahn 1993) in 20 μ l reactions which contained between 0.5 and 1.5 μ l of the various in vitro transcription/translation reactions. In vitro transcription by RNA polymerases I, II, and III was performed as described (Colbert and Hahn 1992). Pol I transcription was assayed by S1 analysis and pol III transcription was assayed by run-off transcription. Pol II transcription employed plasmids which contain the *ACT1*, *HIS4*, or *CYC1* (-52) TATA elements fused downstream of 3 Gal4 binding sites and transcription was assayed by primer extension. Transcription reactions contained between 30 and 120 μ g of nuclear extract protein and 250–500 ng of the indicated template DNAs in 25 μ l reactions. Activated transcription was assayed by including 22 ng Gal4-VP16 in the indicated transcription reactions as described previously (Ranish et al. 1992). Transcription of the yeast U6 gene was performed using pCH6 (Brow and Guthrie 1990) exactly as for tRNA and 5S transcription. Because Brf1 is depleted in our nuclear extracts, 10 ng of recombinant Brf1 was added to all RNA polymerase III transcription reactions. The amount of pol III transcription was proportional to the amount of Brf1 added and there was no difference between *MOT1* and *mot1-1* extracts in pol III transcription when the amount of exogenous Brf1 was varied from 5 to 20 ng per reaction. All transcription reactions were repeated three times and the effect of Mot1 on Pol II transcription was seen in two independently made sets of extracts.

Purification of Mot1C from *E. coli*

E. coli strain BL21 (DE3) (Novagen), containing an integrated copy of the T7 RNA polymerase gene expressed from the induc-

ible *lac* promoter, was transformed with pET15b, pKH9, or pKH13. Cultures (500 ml) were grown at 30°C in TB broth (Sambrook et al. 1989) supplemented with 50 µg/ml of ampicillin to an A_{600} nm of 0.5, and induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 90 min. Cells were collected by centrifugation and resuspended in 3 volumes of buffer A [20 mM Tris-HCl (pH 7.8), 200 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride-HCl (AEBSF, Calbiochem)] and frozen at -70°C. The frozen cells were thawed, and 100 µg/ml lysozyme was added. All subsequent steps were performed at 4°C. Cells were incubated on ice for 30 min and then sonicated for three 10-second pulses, interspersed by cooling on ice. The lysate was then diluted with 8.5 volumes of buffer A and subject to centrifugation at 20,000g for 40 min. A stock of 10% polyethyleneimine (PEI) was added slowly with stirring to a final concentration of 0.1% to the supernatant fraction. After 15 min, the insoluble PEI-DNA complexes were removed by centrifugation at 6000g for 15 min. Solid ammonium sulfate was added slowly to the clarified extract to 40% of saturation. After 30 min of stirring, the extract was subject to centrifugation at 16,000g for 30 min. The resulting supernatant solution was then adjusted by addition of more ammonium sulfate to 60% of saturation. After 30 min of stirring, the Mot1C-containing precipitate was collected by centrifugation at 16,000g for 30 min. The pellet was redissolved in 20 ml of buffer B [20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 5 mM imidazole], and dialyzed against several changes of the same buffer for 12 hr. The dialysate was applied at 20 ml/hr to a column containing 2 ml of iminodiacetate-agarose (His-bind, Novagen) equilibrated in buffer B. The column was washed with 20 ml buffer B, then with 12 ml of buffer B containing 60 mM imidazole. Mot1C was eluted in 4 ml of buffer B containing 500 mM imidazole. The eluate was dialyzed extensively against buffer C [20 mM Tris-HCl (pH 7.8), 200 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol] and frozen in aliquots at -70°C. The preparations of both Mot1C and Mot1C(D1408N) were >90% pure, as judged by SDS-PAGE and staining with Coomassie blue dye.

ATPase assay

Samples of Mot1C (140 ng), Mot1C(D1408N) (140 ng), and an equal volume of the eluate from the vector only control (no detectable protein), or an equal volume of buffer C, were incubated, in duplicate, in a total volume of 20 µl in 50 mM Bis-Tris-propane (pH 6.7), 300 mM NaCl, 2 mM MgCl₂, 100 µg/ml bovine serum albumin, 0.25 mM ATP, 1 µCi [α -³²P]ATP for 30 min at 30°C. Reactions were terminated by addition of an equal volume of 50 mM EDTA. Portions (1 µl) of each reaction mixture were spotted onto the origin of PEI-cellulose thin layer plates (Alltech) and subjected to ascending chromatography in 1 M LiCl, 1 M formic acid (Randerath and Randerath 1964). The amount of ADP formed was quantitated using a PhosphorImager (Molecular Dynamics) and normalized to the total radioactivity applied to each lane.

Sequence analysis of Snf2 family members

Most Snf2/Swi2 family members were identified by BLAST search of the protein and nucleic acid data bases. Selected members were then searched for blocks of related sequences using the MACAW algorithm (Schuler et al. 1991).

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