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Transcriptional Corepression In Vitro: a Mot1p-Associated Form of TATA-Binding Protein Is Required for Repression by Leu3p

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Signals from transcriptional activators to the general mRNA transcription apparatus are communicated by factors associated with RNA polymerase II or the TATA-binding protein (TBP). Currently, little is known about how gene-specific transcriptional repressors communicate with RNA polymerase II. We have analyzed the requirements for repression by the *Saccharomyces cerevisiae* Leu3 protein (Leu3p) in a reconstituted transcription system. We have identified a complex form of TBP which is required for communication of the repressing signal. This TFIID-like complex contains a known TBP-associated protein, Mot1p, which has been implicated in the repression of a subset of yeast genes by genetic analysis. Leu3p-dependent repression can be reconstituted with purified Mot1p and recombinant TBP. In addition, a mutation in the *MOT1* gene leads to partial derepression of the Leu3p-dependent *LEU2* promoter. These in vivo and in vitro observations define a role for Mot1p as a transcriptional corepressor.

Initiation of mRNA synthesis in eukaryotic nuclei is a major point of regulation in the control of gene expression, subject to modulation by a variety of both positive and negative effectors. Current models depict communication between regulatory factors and the general transcription apparatus involving either direct contact of general factors by activators or effects mediated through coactivator molecules (reviewed in reference 41). At least two types of coactivators have been defined, each present in a multiprotein complex and associated with a subset of the general transcription factors. TFIID complexes, which contain the TATA-binding protein (TBP) associated with accessory factors (TAFs), are directly implicated in activation (41). In metazoan systems, direct contacts between several transcriptional activators and individual TAFs have been established (6). In *Saccharomyces cerevisiae*, TFIID complexes have recently been defined (27, 28, 31) and shown to be involved in activation (31). Another class of coactivators has been shown to associate with RNA polymerase II (19, 22). Extensive genetic evidence has linked several components of this complex to transcriptional regulation (reference 19 and references therein).

In contrast to the activation process, molecular mechanisms of gene-specific transcriptional repression remain poorly defined. However, transcriptional repression is an important global regulatory mechanism for large subsets of genes in *S. cerevisiae*. Repression of several metabolic pathways is effected by the *TUP1-SSN6* complex. Genes involved in cell type specification (23), hypoxic growth (reviewed in reference 55), and nutrient utilization (reviewed in reference 33) recruit this repressor complex to promoters of appropriate genes through DNA binding factors unique to each pathway. While the *TUP1-SSN6* complex is the ultimate effector of repression (43), its means of communication with the general transcription machinery remains unknown, although recent genetic analyses implicate communication via factors associated with RNA polymerase II (24, 48). Another global repressor of transcription in *S. cerevisiae* is the protein encoded by the *MOT1* gene. Originally isolated in a screen for factors involved in pheromone-induced transcription, the essential *MOT1* gene is apparently involved in down regulation of many yeast protein-coding genes (9). The recent identification of Mot1p as a TAF (27) and as an ATP-dependent inhibitor of TBP (2, 3) immediately suggested a potential mechanism for Mot1p-mediated repression.

The yeast Leu3 protein is involved in the regulation of genes required for branched-chain amino acid biosynthesis (5, 12) and nitrogen metabolism (17). Leu3p is a useful model transcriptional regulator, as it functions as both an activator and a repressor in vivo (5). Remarkably, the activation function of Leu3p depends entirely on the presence of a small metabolite, α-isopropylmalate (α-IPM), an early intermediate in leucine biosynthesis (5). The repression function of Leu3p is seen either in cells which lack the ability to produce α-IPM (and thus to activate transcription) or in *leu3* mutants which fail to activate transcription. Under these conditions, Leu3p represses reporter gene activity (β-galactosidase fusions) approximately fourfold below the activity observed in cells which lack Leu3p (5). These activation and repression functions can be faithfully reproduced in vitro in a yeast whole-cell transcription extract (38, 39), providing an excellent opportunity for biochemical dissection of the factor requirements and mechanisms of activation and repression. In this work, we have analyzed cofactors necessary for Leu3p-mediated repression in vitro. We find that competence for repression correlates with the presence of a complex, Mot1p-associated form of TBP, similar to metazoan TFIID complexes. Further, purified Mot1p can mediate the repressing signal, defining its function as a corepressor for Leu3p.

MATERIALS AND METHODS

Whole-cell extract production and fractionation. All procedures were performed at 4°C, and all chromatography buffers contained the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 0.4

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μg of bestatin per ml, and 0.35 μg of pepstatin A per ml. Whole-cell extract was produced and fractionated over heparin-Sepharose (Pharmacia) as described elsewhere (46). A representative H300 pool (232 ml; 845 mg of protein) was dialyzed versus D(0) (25 mM Tris HCl [pH 7.9 at 23°C], 10% glycerol, 5 mM EDTA, and 1 mM dithiothreitol, plus protease inhibitors; the number in parentheses gives the millimolar concentration of ammonium sulfate) to a conductance equivalent to that in D(50) and loaded onto a DEAE-Sepharose (Pharmacia) column (7.5 by 2.2 cm) equilibrated in D(50). The column was developed by successive washes with 120 ml each of D(50), D(135), and D(350). Approximately 75 to 80% of the eluted protein from each step (assessed by Coomassie blue spot assay [51]) was pooled and precipitated by the addition of solid ammonium sulfate to 75% saturation. Precipitates were collected by centrifugation at 13,400 rpm in a JA-14 rotor (Beckman) for 20 min. Supernatants were decanted, and the pellets were resuspended in a minimal volume of WCE buffer (47) and dialyzed to equilibrium versus WCE buffer. Fraction volumes and protein content were as follows: D50 pool [i.e., the pool derived by elution with D(50)], 33 ml and 390 mg; D135 pool, 14 ml and 63 mg; D350 pool, 7.2 ml and 32.4 mg.

TBP was resolved on MonoS by the following protocol. D50 (16.5 ml; 195 mg of protein) was filtered through a 0.22-μm-pore-size Gelman acrodisc and applied to a MonoS 10/10 (Pharmacia) column equilibrated in SK(50) (30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol, plus protease inhibitors; the number in parentheses indicates the millimolar concentration of potassium acetate). Protein was eluted by a 46.5-ml wash in SK(50), followed by a linear gradient from SK(50) to SK(1,000) in a volume of 40 ml and a 40-ml wash in SK(1,000). Peak TBP fractions eluted at approximately 0.4 M potassium acetate. For resolution in ammonium sulfate, 16.5 ml (195 mg of protein) of D50 was filtered (as described above) and applied to the same MonoS 10/10 column equilibrated in S(20) (30 mM HEPES [pH 7.5], 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol, plus protease inhibitors; the number in parentheses represents the millimolar concentration of ammonium sulfate). Protein was eluted by a 46.5-ml wash in S(20), followed by a 40-ml linear gradient from S(20) to S(375) and a 40-ml wash in S(375). Peak TBP fractions eluted at approximately 0.12 M ammonium sulfate.

Mot1p was purified as follows. Peak Mot1p-containing fractions from a MonoS column eluted in ammonium sulfate were pooled and dialyzed to equilibrium versus A(1,000) (47), filtered as described above, and loaded on a phenyl-Superose 5/5 column (Pharmacia). Protein was eluted by a 10-ml wash in A(1,000) followed by a linear gradient from A(1,000) to A(0) in 10 ml and a 5-ml wash with A(0). Peak fractions eluted at approximately 0.6 to 0.4 M ammonium sulfate and were pooled. The phenyl pool was then dialyzed to equilibrium versus S(20), filtered as described above, and loaded onto a TSK Heparin 5PW (Toso-Haas) column equilibrated in S(20). The column was developed by a 5-ml wash in S(20), a linear gradient from S(20) to S(375) in 15 ml, and then a 5-ml wash in S(375). Peak Mot1p fractions were at the very end of the gradient, 0.34 to 0.375 M ammonium sulfate. These fractions consisted of highly purified Mot1p (approximately 50% purity as assessed by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) containing a single major impurity of approximately 60 kDa.

Recombinant proteins. Recombinant yeast TBP was purified as described previously (30) from an overproducing strain of *Escherichia coli* (gift of Steve Hahn, Fred Hutchinson Cancer Research Center, Seattle, Wash.). Recombinant yeast TFIIB was purified from an *E. coli* overproducer (pJJ544 in strain BL21[DE3]; gift of E. Maldonado and D. Reinberg, University of Medicine and Dentistry of New Jersey, Piscataway) as follows. Cells were grown in 2 liters of Luria broth plus ampicillin at 37°C to an A_{600} of 0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were collected by centrifugation, washed with cold distilled H_2O, and suspended in 200 ml of DK(100) (25 mM Tris HCl [pH 7.9 at 23°C], 10% glycerol, 5 mM EDTA, and 1 mM dithiothreitol plus protease inhibitors; the number in parentheses indicates the millimolar concentration of potassium acetate). The resulting suspension was lysed by sonication and cleared by centrifugation at 8,000 rpm for 15 min in a JA-14 rotor. TFIIB was purified by chromatography (protocol for DE-52 and Bio-Rex70 columns suggested by E. Maldonado) on 55-ml DE-52 (Whatman) and 12-ml Bio-Rex70 columns in series. Following loading and initial washing with DK(100), the columns were uncoupled and the Bio-Rex70 column was developed by successive washes with 36 ml of DK(100), 60 ml of DK(300), and 60 ml of DK(600). Peak TFIIB fractions (assessed by Coomassie-stained gels) were pooled, dialyzed to equilibrium versus A(1,000), and applied to a phenyl-Superose 10/10 (Pharmacia) column equilibrated in A(1,000). Protein was eluted by a 40-ml wash in A(1,000), a linear gradient from A(1,000) to A(0) in 80 ml, and then a 40-ml wash in A(0). TFIIB eluted at 550 to 450 mM ammonium sulfate. Final purity was >95% according to a Coomassie-stained gel.

Yeast strains for RNA analyses. The isogenic *mot1-1* (YJJ698) and corresponding wild-type (YJJ697) strains used in this study were derived from *mot1-1* (JDY215b; *MAT***a** *ura 3-52 his4-519 leu2-3,112 trp1-1 can1-101 mot1-1*) and isogenic wild-type (JDY194; *MOT1* but otherwise isogenic to JDY215b) strains obtained from Jeremy Thorner. The Thorner strains were converted to leucine prototrophy by transformation (14) with the *Bgl*II fragment of *LEU2* (1).

Transcription assays and RNA analyses. Transcription reactions were carried out as described elsewhere (53). Templates were as described elsewhere (40, 53). For RNA blotting analysis, cells were grown to early log phase under either repressing conditions (medium containing 1% yeast extract, 2% Bacto Peptone, 2% glucose, 2 mM leucine, 1 mM isoleucine, and 1 mM valine) or derepressing and activating conditions (synthetic glucose media [5]). RNA was isolated from yeast, fractionated, immobilized, and probed with the *Bgl*II fragment of *LEU2* as described previously (26). Transcripts were quantified with a PhosphorImager and laser densitometer using ImageQuant software.

Immunoprecipitation and immunoblots. Immunoprecipitations and immunoblots were carried out essentially as described previously (15). Immunoprecipitations with polyclonal sera were performed using 20 μl of fraction 23 from the MonoS column eluted with potassium acetate. Proteins were diluted to a final volume of 100 μl with SK(100), and 1 μl of the appropriate antiserum was added. Following a 1-h incubation on ice, 10 μl of protein A-agarose (Sigma) was added and the tubes were rocked at 4°C for an additional hour. The beads were allowed to settle by gravity, supernatants were removed with a syringe, and the beads were washed once with 100 μl of SK(100).

For immunoprecipitations with the 12CA5 monoclonal antibody, 250 μl of D50 was diluted to 1.4 ml with SK(100) and 40 μl of monoclonal antibody 12CA5 (11) cross-linked to protein A-Sepharose as described elsewhere (15) was added. The resulting suspension was rocked at 4°C for 8 h. Beads were allowed to settle by gravity, supernatants were removed, and the beads were resuspended in 500 μl of SK(100). The resulting suspension was divided into two equal aliquots which were allowed to settle by gravity and subsequently washed twice with 500 μl of either SK(1,000) or S(375). Following the final wash, proteins were eluted with EGTG buffer (50% ethylene glycol, 10% Tween 20, 0.1 M glycine, pH 2.5) and precipitated with acetone as described previously (20). Precipitated proteins were then analyzed by SDS-PAGE and immunoblot.

RESULTS

Resolution of a TBP complex required for response to TFIIA and repression by Leu3p. General transcription factor IIA (TFIIA) has been described as an anti-inhibitor which antagonizes negative regulators found in the native TFIID fraction (8, 29). We reasoned that a requirement for TFIIA might reflect the presence of factors involved in repression. Therefore, we began to study gene-specific repression in vitro by first defining a TFIIA-responsive transcription system (46, 47). Using chromatography on DEAE-Sepharose (see Materials and Methods), we derived three fractions from this extract which were required for transcription in addition to TFIIA (Fig. 1), with TFIIA exerting a stimulatory effect. Replacement of one fraction (D50; Fig. 1) with purified recombinant TBP and TFIIB (rTBP and rTFIIB) resulted in TFIIA independence (Fig. 2A, compare lanes 1 and 2 with lanes 3 and 4). While transcription driven by rTBP and rTFIIB no longer responded to TFIIA, it was fully responsive to the model transcriptional activator Gal4-VP16 (Fig. 2B). Consistent with our initial hypothesis, this reconstituted system lost the ability to support repression by Leu3p. As shown in Fig. 3, addition of Leu3p (in the absence of α-IPM) to a whole-cell transcription extract (Fig. 3, lanes WCE), represses transcription two- to threefold from a template containing a UAS_{LEU} relative to transcription from a UAS^- template. When transcription was reconstituted with rTBP and rTFIIB (Fig. 3, lanes Recon.), transcript levels from the UAS_{LEU} template are equal to or higher than those from the UAS^- template. This equimolar transcription from the two templates was identical to that observed in the absence of added Leu3p (data not shown; see also Fig. 4B). Additionally, unlike the case with Gal4-VP16, activation by Leu3p in the presence of α-IPM is two- to threefold lower in the reconstituted transcription system (Fig. 3).

To determine specific factors required for TFIIA sensitivity and transcriptional repression, the D50 pool was further fractionated by gradient elution on a MonoS column (Fig. 1; Materials and Methods). We found that reconstitution of TFIIA-stimulated transcription was sensitive to the nature of the eluting salt. TFIIA was required for optimal activity when transcription was reconstituted with TBP-containing fractions eluted in potassium acetate but not for similar fractions eluted with ammonium sulfate (Fig. 4A, compare lanes 1 and 2 with

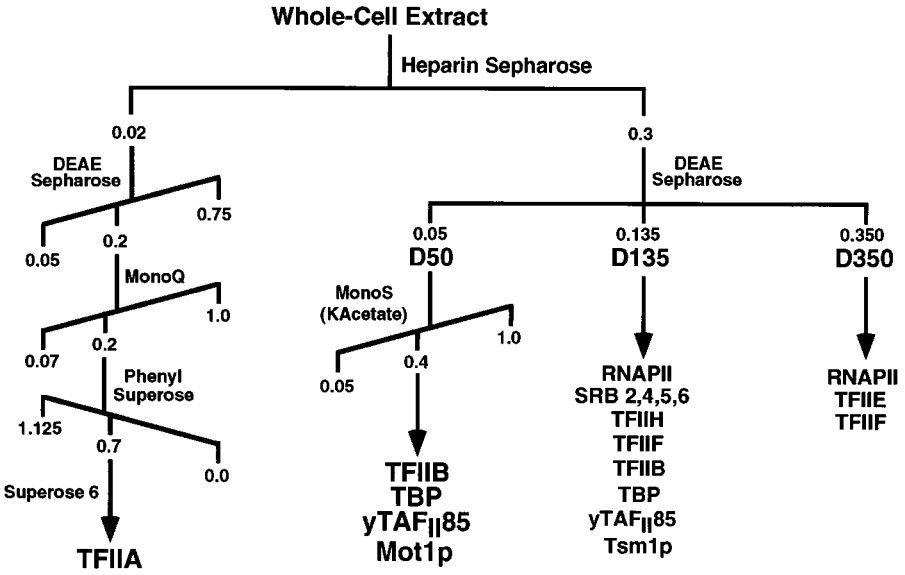

FIG. 1. Fractionation scheme. Chromatographic steps utilized in generation of fractions used in this study are summarized in the flow chart. Molarity of ammonium sulfate or, where indicated, potassium acetate used in the fractionation is indicated. Details of the purification of TFIIA can be found in reference 46. Identification of factors not described in the text was by immunoblot or transcription reconstitution assays (reference 36 and data not shown).

lanes 3 and 4). In fact, ammonium sulfate-eluted TBP was indistinguishable from the recombinant factor in these experiments, while the potassium acetate-eluted fractions were stimulated approximately 10-fold by TFIIA (Fig. 4A, data not shown). Using reconstitution experiments, we also found that only potassium acetate-eluted TBP fractions retained competence for repression by Leu3p (Fig. 4B). The three- to fourfold repression that we observed in these reactions was similar to that previously measured in an unfractionated whole-cell extract (Fig. 3; see also reference 40). While transcription from the UAS_{LEU} and UAS^{-} templates is not equimolar when transcription is reconstituted with these MonoS fractions (as it is with the recombinant factors), we have observed similar effects using excessive amounts of whole-cell extract protein in transcription assays on UAS-containing templates (52).

Composition and physical characterization of the TBP complex. We have been unable to recreate the properties of the potassium acetate-eluted TBP by mixing fractions from the ammonium sulfate elution, nor have we been able to purify the TBP complex further by conventional means while retaining the TFIIA responsiveness and repression by Leu3p. These observations are consistent with a multiprotein complex that is readily dissociated, reminiscent of the metazoan TFIID complex. To identify other components of this putative complex, we analyzed the gradient fractions by immunoblot. We found that $yTAF_{II}85$, the homolog of *Drosophila* $TAF_{II}80$ (31) and $yTAF_{II}180$, also known as Mot1p (27), were both found in the D50 fraction (Fig. 1) and coeluted with TBP under conditions that maintained responsiveness to TFIIA and Leu3p (Fig. 5). The elution pattern of Mot1p and $yTAF_{II}85$ changed depending on the eluting salt. Both proteins showed peaks overlapping that of TBP when eluted with potassium acetate, but these overlaps were reduced or absent upon elution in ammonium sulfate (Fig. 5). These results indicated that potassium acetate might stabilize protein-protein interactions between Mot1p, $yTAF_{II}85$, and TBP in a TFIID-like complex.

To examine the nature of the interactions between TBP, $yTAF_{II}85$, and Mot1p, we performed immunoprecipitations. We first asked whether all three proteins existed in a single complex by performing immunoprecipitations with the antisera used in Fig. 5. As shown in Fig. 6A, antisera specific for Mot1p and $yTAF_{II}85$ both efficiently coprecipitate TBP. The anti-Mot1p serum coprecipitated some $yTAF_{II}85$, and the converse was also true (Fig. 6A, lanes 1 and 2). The anti-TBP serum coprecipitated both Mot1p and $yTAF_{II}85$ (Fig. 6A, lane 3). Parallel experiments with preimmune serum from the same rabbit which produced the anti-TBP antibody failed to coprecipitate any of these species (Fig. 6B and data not shown).

These experiments support the hypothesis that chromatography in potassium acetate preserves the integrity of a single, multiprotein complex minimally containing TBP, $yTAF_{II}85$, and Mot1p. However, the recovery of Mot1p in all the precipitations is less striking than that of the other components. To confirm these results and to extend the characterization of this unstable complex, we have precipitated the proteins from the input to the MonoS chromatography (D50 fraction; Fig. 1) and washed them with different buffers to assess their effects on stability. To facilitate these experiments, we obtained a yeast strain (YTW33; kind gift of P. A. Weil) whose sole source of TBP is a plasmid-borne *SPT15* gene tagged at its amino terminus with an epitope from the influenza virus hemagglutinin protein (HA1) (11). While this construct expresses TBP at slightly elevated levels compared with levels expressed by the chromosomal locus, fractions derived from this strain are qualitatively indistinguishable in transcription reconstitution assays from fractions derived from wild-type yeast strains (data not shown). We found that when immune complexes were formed in and washed with buffers containing potassium acetate, TBP, $yTAF_{II}85$, and Mot1p coprecipitated from the D50 fraction (Fig. 6C, lane 2). The interaction between Mot1p and the other components of the complex appears to be considerably weaker than the TBP-$yTAF_{II}85$ interaction (compare the ratio of Mot1p in Fig. 6B, lane 1 versus lane 2 with the same ratio for $yTAF_{II}85$). When immune complexes were formed in potassium acetate buffer and washed in ammonium sulfate, little or no detectable Mot1p remained in the complex (Fig. 6B, lane 3). Recovery of $yTAF_{II}85$ was also reduced by the ammonium sulfate wash (compare the ratio of TBP to $yTAF_{II}85$ in Fig. 6B,

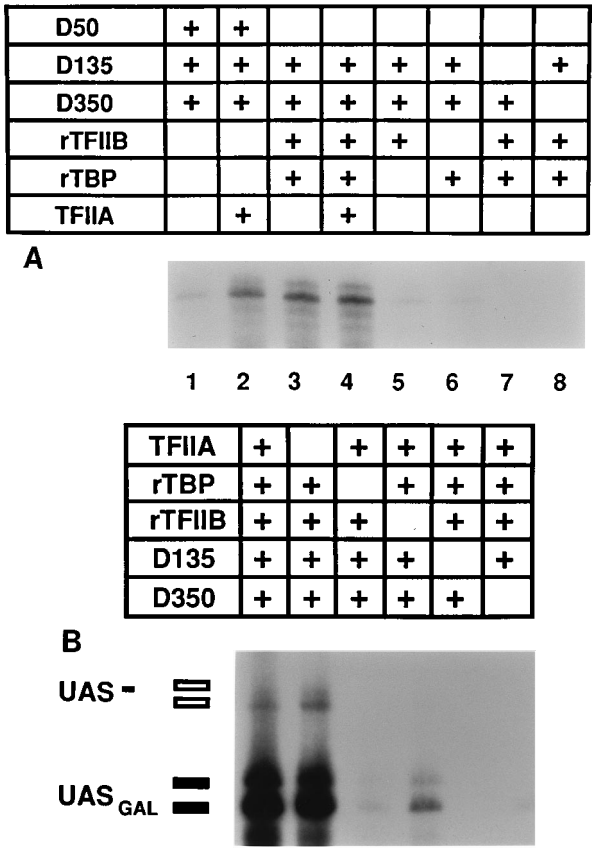

FIG. 2. Reconstitution of the fractionated whole-cell extract reveals a dependence on TFIIA which is lost with recombinant forms of TBP and TFIIB. (A) Transcription reactions were performed as described in Materials and Methods. The following amounts of each fraction were used: TFIIA (Superose 6 fraction), 120 ng; D50, 16.8 μg; rTBP, 25 ng; rTFIIB, 100 ng; D135, 13.5 μg; D350, 13.5 μg. Gal4-VP16 (20 ng) was present in each reaction. The template contained a UAS_{GAL}. (B) Transcription reactions were performed as described in Materials and Methods. Open bars denote the mobility of products derived from a template lacking a UAS; closed bars denote the mobility of products derived from a template bearing a UAS_{GAL}. The following amounts of each fraction were used: TFIIA (Superose 6 fraction), 120 ng; rTBP, 100 ng; rTFIIB, 200 ng; D135, 13.5 μg; D350, 13.5 μg; Gal4-VP16, 20 ng.

lane 3, with that in lane 2). Inclusion of the epitope peptide in the immunoprecipitation reaction abolishes precipitation of both Mot1p and $yTAF_{II}85$, precluding the possibility of a nonspecific interaction with the solid-phase matrix (Fig. 6B, lane 4).

Mot1p can mediate repression by Leu3p. We have demonstrated that the presence of Mot1p correlates with competence for repression and TFIIA responsiveness (Fig. 1; see also Discussion), consistent with its previously described genetic and biochemical properties. To establish the role of Mot1p in repression by Leu3p we purified Mot1p from yeast and directly tested its ability to reconstitute repression in the presence of purified recombinant TBP (Fig. 7). As described above and shown in Fig. 7, without added Mot1p, Leu3p had little or no effect on transcription of a UAS_{LEU} template. In the absence of added Leu3p, purified Mot1p resets basal transcription from templates containing or lacking a UAS_{LEU} to a lower level. The reduction of the basal level on the UAS_{LEU} template (Fig. 7) and on the UAS^- template (data not shown) is consistent with the results of Auble and coworkers, who also observed decreased levels of basal transcription (ranging from 5- to 15-fold) using nuclear extracts derived from wild-type as compared to *mot1-1* mutant cells (3). In our experiments, the combination of Mot1p and Leu3p decreased transcription from the UAS_{LEU} template an additional fourfold (Fig. 7), which is similar to the levels of repression seen in the whole-cell (40) and fractionated extracts (Fig. 3). We found that the addition of Leu3p had no effect on the Mot1p-reduced levels of transcription from the UAS^- template (data not shown), ruling out a nonspecific but additive effect of the two repressors on the UAS_{LEU} template.

Although these data establish an important role for Mot1p as a corepressor, they do not rule out the possibility that additional TAF_{II}s are also important in this process. In fact, although we can reconstitute repression with these purified proteins, we were unable to reproduce this phenomenon simply by mixing the more complex fractions containing these factors from the MonoS chromatography (see Fig. 4 and text above). As the purified Mot1p preparation is more concentrated than the MonoS fractions (three- to fivefold [data not shown]), we propose that TAF-TAF interactions are crucial for the productive interaction of TBP and Mot1p at lower factor concentrations.

The *mot1-1* mutation partially derepresses a Leu3p-regulated gene. If Mot1p mediates repressing signals from Leu3p, then a mutation in *MOT1* should affect transcription of a Leu3p-dependent promoter. As described by Davis and coworkers (9), a mutation (*mot1-1*) in the essential *MOT1* gene derepresses the expression of several yeast genes two- to threefold (on the basis of analyses of transcript levels). Genetic analysis of the repression function of Leu3p, evident in cells unable to synthesize α-IPM or in activation-deficient *leu3* mutants (5), predicts an approximately fourfold value for repression (based on measurements of reporter gene activity). To dissect the effects of Mot1p on Leu3p-driven repression in vivo, we have examined transcription from the Leu3p-dependent *LEU2* gene using the adjacent $tRNA_3^{LEU}$ (tRNA transcription is unaffected by the *mot1-1* mutation [3]) as an internal standard. We grew cultures in media which should result in either transcriptional repression (rich media supplemented with high levels of leucine, isoleucine, and valine) or derepres-

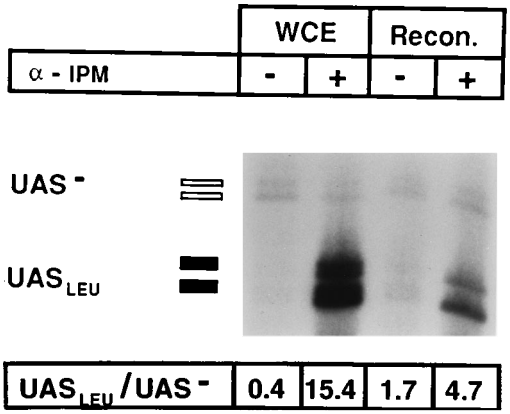

FIG. 3. Transcription reconstituted with rTBP and rTFIIB does not support repression by Leu3p. Transcription reactions were performed as described in Materials and Methods. WCE, whole-cell transcription extract; Recon., transcription reconstituted with rTBP and rTFIIB. Open bars denote transcripts from a template lacking a UAS; closed bars denote transcripts from a template bearing a UAS_{LEU}. The following amounts of each fraction were used: WCE, 75 μg; rTBP, 100 ng; rTFIIB, 200 ng; D135, 13.5 μg; D350, 13.5 μg. All reaction mixtures contained 4 μl of purified Leu3p (38). Indicated reaction mixtures contained α-IPM at a final concentration of 1 mM.

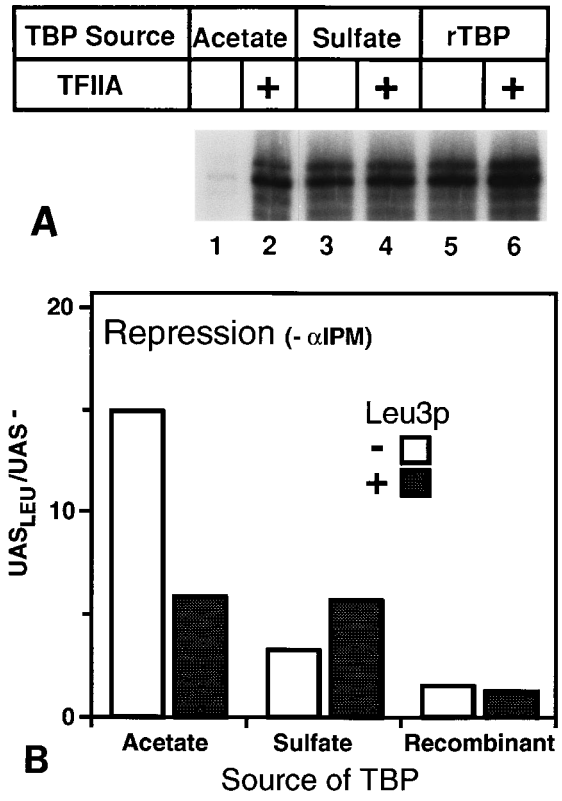

FIG. 4. Elution conditions dramatically alter the transcriptional properties of yeast TBP. (A) Transcription reactions were carried out using the following amounts of each fraction: D135, 13.5 μg; D350, 13.5 μg; rTFIIB, 90 ng; rTBP, 50 ng; TFIIA, 120 ng; potassium acetate TBP fraction, 6 μg; ammonium sulfate TBP fraction, 3.6 μg; Gal4-VP16, 20 ng. All reactions were run on the same gel; the lanes have been rearranged for clarity of presentation. (B) Transcription reactions were carried out using the templates described in the legend to Fig. 3. Factor amounts are given above. The bars labeled "Recombinant" represent results obtained in reactions reconstituted with purified rTBP and rTFIIB as described in Materials and Methods and for panel A. Radioactivity in specific transcripts was quantified with a PhosphorImager. The bar graph depicts the ratio (after normalization for transcript length) of transcripts from the two templates in each reaction. Unlike reactions using whole-cell extracts in which the ratio of transcription from the UAS_{LEU} relative to the UAS^- template is approximately 1, we reproducibly find that the UAS_{LEU} template is preferred by the complex reconstituted reactions. We observe similar effects on a UAS_{GAL} template using very high levels of protein from a Gal4 deletion whole-cell extract (52).

sion and activation (synthetic media lacking leucine, isoleucine, and valine [5]). Consistent with the results of Brisco and Kohlhaw (5), we reproducibly observed four- to fivefold higher levels of *LEU2* mRNA under derepressing conditions (Fig. 8). The *mot1-1* mutation had no effect on the derepressed or activated levels of transcription in these experiments (Fig. 8), precisely as Auble and colleagues described for expression of several activated (UAS-driven) reporter constructs (3). In contrast, when grown under repressing conditions, *LEU2* transcription in the *mot1-1* mutant is elevated (1.7-fold) over its level in wild-type cells (Fig. 8). In several experiments, this derepression varied from 1.3- to 1.7-fold (data not shown). This modest effect on repression was not unexpected on the basis of the prior genetic studies of Mot1p and Leu3p. Additionally, this genetic background (wild-type Leu3p and competence for α-IPM synthesis) is suboptimal for observation of the repressing effect of Leu3p. Further complicating this analysis, *LEU2* transcription is also subject to general amino acid control exerted by Gcn4p (5). These results do, however, support an in vivo role for *MOT1* in mediating repressing signals from *Leu3* and confirm the results of our in vitro experiments with purified Mot1p and Leu3p.

DISCUSSION

Transcriptional repression is a major form of eukaryotic gene regulation. Chromatin structure represses transcription of many mRNA encoding genes via direct promoter occlusion (reviewed in reference 25). Some DNA-binding proteins initially identified as activators of transcription have subsequently been characterized as repressors in certain contexts (reviewed in reference 42). The majority of promoters analyzed in detail contain both positive and negative regulatory elements. Despite the importance of repression in gene regulation, little is known about the mechanisms by which specific negative control elements transduce repressing signals to the general transcription apparatus. If repression is as mechanistically complex as activation, many different pathways will undoubtedly be discovered. Transcriptional activation potentially involves communication with every component of the transcriptional machinery. Direct contact between activators and TBP, TFIIB, TFIIF, and TFIIH has been reported (reviewed in reference 41). Indirect contact mediated by coactivators either in the holoenzyme form of RNA polymerase II (19, 22) or in a TFIID complex has been demonstrated (reviewed in reference 41). Recent reports have described mechanisms of eukaryotic re-

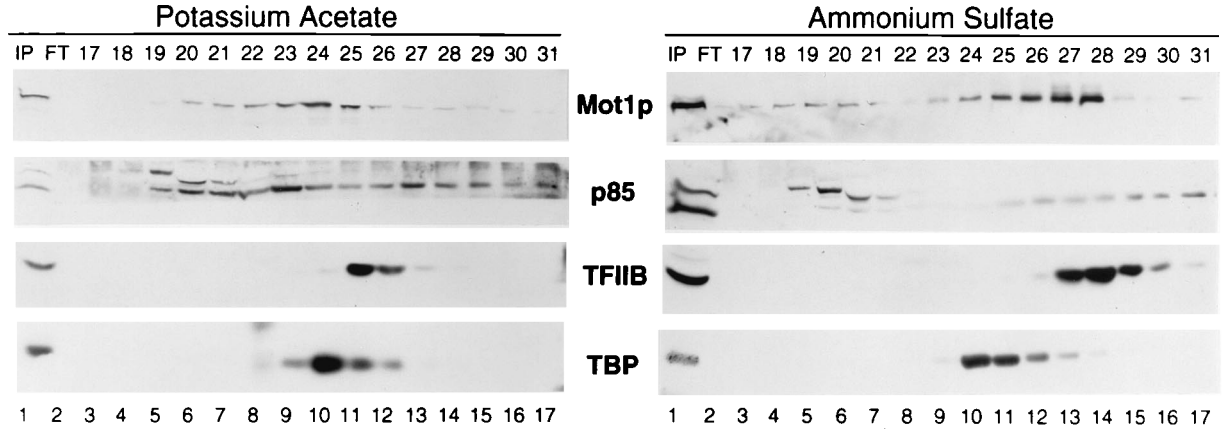

FIG. 5. The eluting salt affects the coelution of TBP and TAF_{II}s. The D50 fraction (Fig. 1) was chromatographed on MonoS as described in Materials and Methods with ammonium sulfate or potassium acetate as the eluting salt. The indicated column fractions (10 μl) were analyzed by immunoblot.

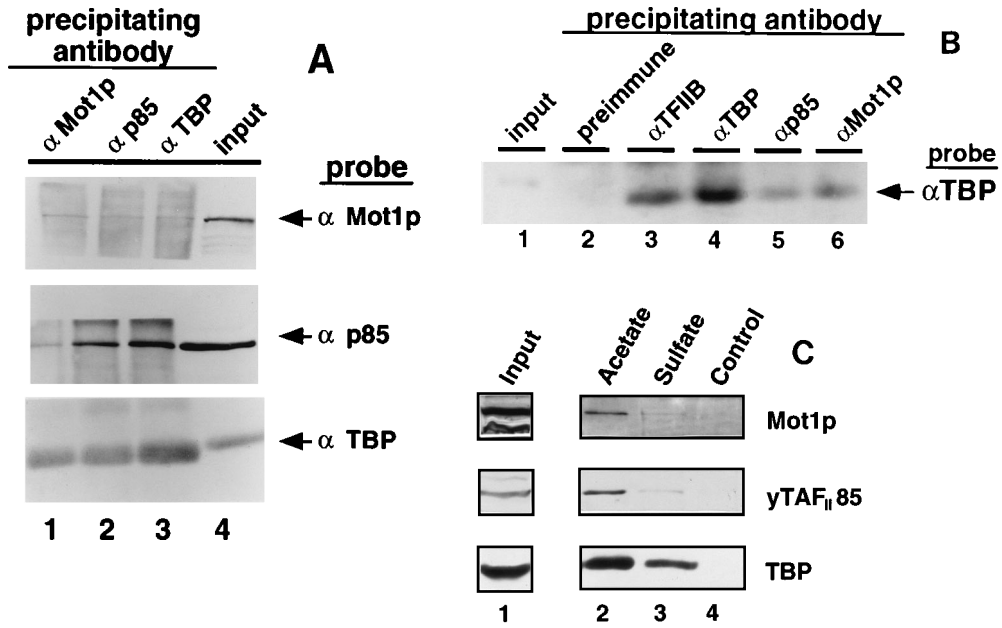

FIG. 6. Identification of a complex containing TBP, Mot1p, and $TAF_{II}85$. (A) Immunoprecipitations from MonoS fraction 23 (potassium acetate elution; Fig. 1 and 5) with the indicated polyclonal antibodies were performed as described in Materials and Methods. Precipitated proteins were analyzed by SDS-PAGE and immunoblot. The indicated polyclonal sera were used to detect precipitated species. (B) Immunoprecipitations from MonoS fraction 23 (Fig. 1 and 5) with preimmune serum (lane 2) and the indicated polyclonal sera were performed as for panel A. (C) Immune complexes (using the 12CA5 monoclonal antibody [11]) were formed from the D50 fraction in potassium acetate-containing buffers and washed with either potassium acetate- or ammonium sulfate-containing buffers as described in Materials and Methods. Proteins were eluted from the solid-phase matrix as described in the text and analyzed by immunoblot. The control experiments (lane 4) contained the epitope peptide in the immunoprecipitation reaction and were washed with potassium acetate-containing buffers.

pressors which function by promoter occlusion (49) or through interactions with TFIIE (35) or TBP-TFIID (4, 44). In this work, we have established a pathway for negative regulation by a gene-specific repressor, the Leu3 protein. Leu3p binds constitutively to the UAS_{LEU} and switches from a repressor to an activator in the presence of a small metabolite (5). We have demonstrated that repression by Leu3p requires a complex form of yeast TBP with properties similar to those of the TFIID factor characterized in other eukaryotic systems. This complex is also responsive to TFIIA, providing further evidence for the role of this essential transcription factor in global gene expression and regulation.

We found two known TAF_{II}s in the repression-competent TFIID-like complex. The ability to mediate repression is lost under conditions which dissociate these factors from TBP. One factor, $yTAF_{II}180$, also known as Mot1p, can reconstitute Leu3p repression in vitro with recombinant TBP. We also detected $yTAF_{II}85$ in the TBP complex. Immunoprecipitation experiments confirmed that Mot1p, $yTAF_{II}85$, and TBP are all associated in a complex (Fig. 6), confirming recent reports from Poon and coworkers (27, 28). Additionally, we have observed a high-molecular-weight complex that contains Mot1p and $yTAF_{II}85$ but lacks TBP (data not shown). We interpret these data as evidence that Mot1p may interact with one or more of the TAF_{II}s in addition to its known interaction with TBP (2, 3).

In contrast to previous reports (27, 45), we detected no $yTAF_{II}150$, also known as Tsm1p, in the repression-competent TFIID-like fractions. Instead we observed that about 5% of the TBP and $yTAF_{II}85$ cofractionated with all of the detectable Tsm1p elsewhere in our fractionation scheme (Fig. 1). This same fraction also contains the components of the RNA polymerase II-associated mediator complex (19, 22) and may therefore contain a second TFIID-like complex. The mutual exclusivity of Tsm1p and Mot1p is the most striking characteristic of these two species and supports some previous work by Poon and coworkers (27). Our observation that the Mot1p-containing fractions were not required for activation by Leu3p or GAL4-VP16 (Fig. 2 and 3) is consistent with previous reports that either a Tsm1p-containing TAF_{II} preparation (27) or the mediator complex (19, 22) is sufficient to confer activation in vitro.

Using immunoprecipitation, Poon and coworkers found that only 3 to 5% of TBP from a whole-cell extract is Mot1p associated (27). In contrast, in our biochemical fractionation, we have observed that greater than 90% of TBP cofractionates

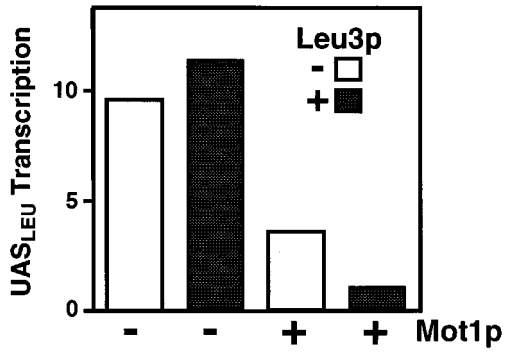

FIG. 7. Purified Mot1p functions as a corepressor for Leu3p. Transcription reactions were performed as for Fig. 4B on templates containing or lacking a Leu3p binding site (UAS_{LEU}), with or without Mot1p and Leu3p purified from yeast as indicated. Transcripts from the UAS_{LEU} template were quantified by laser densitometry of autoradiograms as described in Materials and Methods. Transcript abundance for each template was normalized to that in the reactions containing both Leu3p and Mot1p.

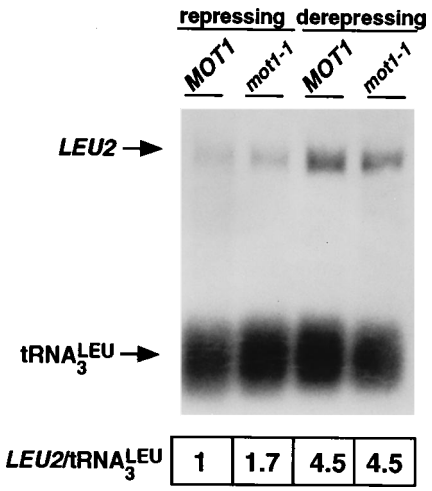

FIG. 8. A Leu3p-dependent transcript is derepressed in the *mot1-1* mutant strain. RNA was isolated from isogenic wild-type (YJJ697) and *mot1-1* (YJJ698) strains, and transcription from the *LEU2* and $tRNA_3^{LEU}$ genes was analyzed as described in Materials and Methods. Repressing and derepressing refer to growth conditions resulting in repression (rich media supplemented with leucine, isoleucine, and valine) or derepression (synthetic media lacking leucine, isoleucine, and valine) of *LEU2* transcription. Transcript abundance was quantified with a PhosphorImager. The ratio of the transcripts from the two templates was calculated for each lane and was normalized to the wild-type strain grown under repressing conditions.

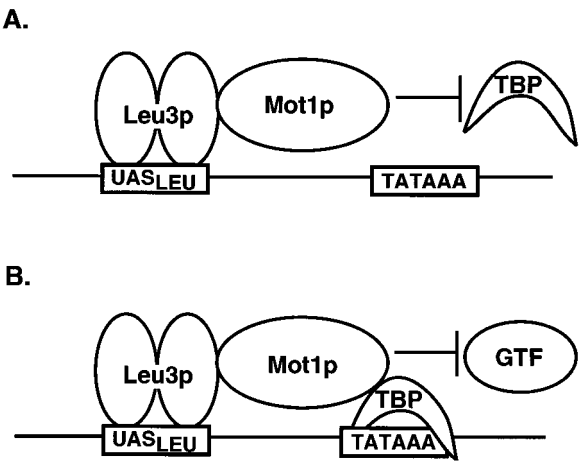

FIG. 9. Models for Mot1p-mediated transcriptional repression. Features of the two models are described in the text. GTF, general transcription factor.

with Mot1p. This seeming contradiction is likely due to differences in protocol or to differences in the abundance or stability of Mot1p- or Tsm1p-containing complexes. Although a Mot1p homolog has not been identified in other systems, TFIID preparations from *Drosophila* (10, 21, 45, 50) and human (7, 16, 34, 54) cells contain variable amounts of the Tsm1p homolog $TAF_{II}150$. In contrast to the variations in Mot1p and Tsm1p content, the remaining polypeptide composition of all reported TFIIDs is very similar. Tsm1p and Mot1p may represent factors peripherally associated with the core TAF_{II} complex, which can modulate its activity in response to different regulatory factors. Heterogeneity of TAF content has also been observed for human $TAF_{II}30$ and $TAF_{II}150$ (18), where complexes of different composition respond to different activators.

The identification of Mot1p as a transcriptional corepressor is consistent with its previous genetic characterization as a repressor of a subset of yeast promoters (9). In addition, in vitro experiments have demonstrated that Mot1p can remove TBP from DNA in an ATP-dependent reaction (2). Since simple promoter occlusion has been ruled out as a mechanism of repression by Leu3p (32, 39), we can consider at least two possible mechanisms for template-specific repression (Fig. 9). Both mechanisms include the prediction that local concentrations of Mot1p may be increased at the UAS_{LEU} promoter through protein-protein interactions with the repressing form of Leu3p. In this regard, it is intriguing to note that the amino-terminal region of Mot1p contains several potential tetratricopeptide motifs (9), a motif probably involved in protein-protein interactions and found in several transcription factors, including the repressor *SSN6* (37). Mot1p may interact with many different proteins through individual tetratricopeptide motifs much as Tup1p interacts with its many partners via the repeating WD40 motif (43). Mot1p could then destabilize the TBP-DNA interaction as shown by Auble and colleagues (2, 3) (Fig. 9A). In an alternative model (Fig. 9B), a stable TBP-Mot1p-DNA complex may prohibit subsequent association of a downstream general transcription factor on the UAS_{LEU} promoter. Mot1p forms stable ternary complexes with TBP at some promoters, including the Leu3p-regulated *LEU2* promoter (2, 5). Perhaps Leu3p promotes the formation of a stable TBP-Mot1p-DNA complex like that described for TBP and HMG1, which then prevents binding of TFIIB and subsequent preinitiation complex formation (13). While the work of Auble and colleagues (2, 3) supports the model depicted in Fig. 9A, we cannot currently rule out the possibility described in Fig. 9B. Resolution of these possibilities will require further definition of the factors required for repression and the steps that they affect in initiation.

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