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Cellular Stress Alters the Transcriptional Properties of Promoter-Bound Mot1-TBP Complexes

Joseph V. Geisberg and Kevin Struhl*
Department of Biological Chemistry
and Molecular Pharmacology
Harvard Medical School
Boston, Massachusetts 02115

Summary

Mot1 associates with transcriptionally active promoters, and it directly affects transcriptional activity in a positive or negative manner, depending on the gene. As determined by sequential chromatin immunoprecipitation, Mot1 co-occupies promoters with TBP, but not with TFIIB, TFIIA, or Pol II when cells are grown in normal conditions. This strongly suggests that the Mot1-TBP complex is transcriptionally inactive, and hence is in dynamic equilibrium with transcriptionally active forms of TBP. Surprisingly, in response to heat shock and other forms of environmental stress, Mot1 co-occupies promoters with TFIIB and elongation-competent Pol II, but not with TFIIA. This suggests that functional preinitiation complexes can contain Mot1 instead of TFIIA in vivo. Thus, Mot1-TBP complexes can exist in active and inactive forms that are regulated by environmental stress.

Introduction

In yeast cells, the TATA binding protein (TBP) is generally required for transcription by RNA polymerase II (Cormack and Struhl, 1992), and the level of TBP association with promoters is strongly correlated with transcriptional activity (Kuras and Struhl, 1999; Li et al., 1999). Yeast TBP exists as the free polypeptide (Hahn et al., 1989) and as a component of multiprotein complexes that affect Pol II transcription such as TFIID, Mot1-TBP, NC2-TBP, and Not-Ccr4-TBP (Lee and Young, 1998). TFIID, a complex comprising TBP and 14 TBP-associated factors (TAFs), is recruited to ribosomal protein promoters by a Rap1-containing activator (Li et al., 2002; Mencia et al., 2002), and it is important at several promoters with weak TATA elements (Moqtaderi et al., 1996; Shen and Green, 1997; Mencia et al., 2002). However, a TAF-independent form(s) of transcriptionally active TBP predominates at many other promoters, and it is preferentially recruited to promoters by most yeast activators (Kuras et al., 2000; Li et al., 2000, 2002; Mencia et al., 2002).

In principle, the TAF-independent form of transcriptionally active TBP could be the free subunit and/or a TBP-containing complex. Two TBP-associated proteins, Mot1 and NC2, were initially characterized as general negative regulators of TBP function (reviewed by Lee and Young, 1998). However, both factors can positively affect transcription from certain promoters (Collart, 1996; Madison and Winston, 1997; Lemaire et al., 2000; Geisberg et al., 2001; Andrau et al., 2002; Dasgupta et al., 2002; Geisberg et al., 2002). The Bur6 subunit of NC2 associates with transcriptionally active promoters (Geisberg et al., 2001; Creton et al., 2002), and the Bur6:TBP occupancy ratio is particularly high at promoters where Bur6 is important for normal levels of transcription (Geisberg et al., 2001). Promoter association of the Ydr1 subunit of NC2 correlates with transcriptional repression of certain promoters, and association of the Bur6 and Ydr1 subunits with each other is regulated, suggesting the existence of multiple NC2 complexes with different transcriptional functions (Creton et al., 2002). Thus, it is unclear whether NC2 or the Bur6 subunit is part of a transcriptionally active form of TBP in vivo.

Mot1 stably associates with the TBP-TATA complex, and it can dissociate TBP from DNA in an ATP-dependent manner (Auble et al., 1994; Gumbs et al., 2003). Mot1 inhibits transcription in vitro in a manner that can be blocked by TFIIA (Auble and Hahn, 1993; Auble et al., 1994; Chicca et al., 1998). In vivo, Mot1 removes TBP, but not TBP complexes, from inappropriate genomic locations (Li et al., 1999; Geisberg et al., 2002), suggesting that free TBP is the physiologically relevant substrate for Mot1 (Geisberg et al., 2002). It was originally suggested that positive transcriptional effects of Mot1 were due to redistribution of TBP between promoter and nonpromoter regions (Collart, 1996; Madison and Winston, 1997; Muldrow et al., 1999). However, Mot1 associates with transcriptionally active promoters in vivo, and it is rapidly recruited by sequence-specific activator proteins to inducible promoters in response to external stimuli (Geisberg et al., 2002). Furthermore, the Mot1:TBP occupancy ratio at both Mot1-stimulated and Mot1-inhibited promoters is high relative to typical promoters (Dasgupta et al., 2002; Geisberg et al., 2002), and the Mot1 ATPase activity is required for the both the positive and negative effects of Mot1 on transcription (Dasgupta et al., 2002). These results strongly suggest that Mot1 directly affects transcriptional activity in a positive or negative manner, depending on the gene.

There are two mechanisms by which Mot1 associates with Pol II promoters in a manner that strongly correlates with transcriptional activity. In one model, the Mot1:TBP complex is a TAF-independent form of transcriptionally active TBP that forms normal preinitiation complexes and is recruited, directly or indirectly, by activator proteins. In this view, the Mot1-TBP complex is analogous to TFIID. However, its specificity for core promoters and/or activators might differ from that of TFIID, as the Mot1-TBP complex possesses DNA binding properties that differ from those of free TBP when assayed in vitro in the presence of a non-hydrolysable analog of ATP (Gumbs et al., 2003). In an alternative model, the Mot1-TBP complex is not transcriptionally active per se, but Mot1 simply associates with free TBP at accessible TATA elements in the absence of a functional preinitiation complex. In this model, the Mot1-TBP complex is in a dynamic equilibrium with transcriptionally active forms of TBP. In this regard, Mot1 inhibits the associa-

*Correspondence: kevin@hms.harvard.edu

tion of NC2 with promoters, indicating that the Mot1-TBP and NC2-TBP complexes compete for promoter occupancy in vivo (Geisberg et al., 2002). In addition, Mot1 association with promoters is largely independent of the Srb4 component of the Mediator complex, and Mot1 associates with highly active Pol III promoters (Geisberg et al., 2002).

Here, we use sequential chromatin immunoprecipitation to analyze whether Mot1 co-occupies promoters with various components of the Pol II transcription machinery. Under normal growth conditions, we find that Mot1 does not co-occupy promoters with TFIIB, TFIIA, or Pol II, strongly suggesting that the Mot1-TBP complex is inactive, and hence in equilibrium with active complexes. Surprisingly, under conditions of environmental stress (heat shock, excess copper, high osmolarity), Mot1 co-occupies promoters with TFIIB and Pol II, but not with TFIIA, suggesting that Mot1 can replace TFIIA in preinitiation complexes. These observations suggest that Mot1 exists in transcriptionally active or inactive forms depending on the environmental conditions.

Results

Genome-Wide Location of Mot1

Although Mot1 association with promoters correlates strongly with TBP occupancy and transcriptional activity, Mot1-regulated promoters show unexpectedly high levels of Mot1 occupancy (Dasgupta et al., 2002; Geisberg et al., 2002). To identify specific classes of genes with high levels of Mot1 occupancy, we performed genome-wide location analysis, a procedure that combines chromatin immunoprecipitation with hybridization on DNA microarrays representing the near-complete repertoire of intergenic sequences (Ren et al., 2000; Iyer et al., 2001; Ng et al., 2002). When cells were grown at 30°C, we found significant Mot1 occupancy (>2-fold over the median; see Experimental Procedures) at 119 intergenic regions, of which 107 were upstream of Pol II-transcribed genes. The median transcription of Mot1-bound promoters is 7-fold higher than the genome-wide median (14.7 versus 2 molecules/hour; $P < 6 \times 10^{-13}$), and ribosomal protein genes are significantly overrepresented ($P < 1 \times 10^{-10}$). Upon a 15 min heat shock at 39°C, significant Mot1 occupancy was observed at 231 Pol II-regulated promoters, with genes involved in the stress response being highly overrepresented ($P < 6 \times 10^{-19}$). In contrast, ribosomal protein genes are no longer significantly overrepresented, as expected from their reduced transcription in response to stress. Thus, in accord with previous results, Mot1 association at promoters correlates strongly with transcriptional activity. We were unable to find any statistically significant group of genes that are poorly expressed yet have meaningful Mot1 occupancy under either 30°C or heat shock conditions.

Co-Occupancy of TBP, TFIIB, TFIIA, and Pol II at Promoters In Vivo

Although standard chromatin immunoprecipitation (ChIP) experiments can determine the relative levels of different proteins at genomic regions, they do not address whether two proteins simultaneously occupy a given DNA sequence. The observation that two proteins associate with a given genomic region might reflect co-occupancy, but it also could indicate that the two proteins associate with different (and perhaps mutually exclusive) populations of DNA molecules. Sequential ChIP enables the detection of simultaneous occupancy (co-occupancy) of two different proteins at any genomic region of interest (Scully et al., 2000; Chaya et al., 2001; Proft and Struhl, 2002; Soutoglou and Talianidis, 2002). In this procedure, crosslinked protein-DNA complexes from living cells are immunoprecipitated by one antibody, eluted, and then immunoprecipitated by a second antibody. The resulting material and control samples from the corresponding single immunoprecipitations are analyzed by quantitative PCR in real time, and the data are presented as fold enrichments for a given promoter region as compared to an internal fragment of the *POL1* coding region in the same sample.

Sequential ChIP experiments have three possible outcomes (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/14/4/479/DC1 for a detailed theoretical analysis). First, "complete co-occupancy" takes place when two factors always localize to the same DNA fragment; neither factor is found on the DNA fragment in the absence of the other. If two proteins always co-occupy a given promoter, the fold enrichment in a sequential ChIP experiment should be equivalent to the product of the fold enrichments of the individual ChIP experiments, and the order of the immunoprecipitations should not matter. Second, "no co-occupancy" occurs when the two proteins associate with the same genomic region in vivo but on mutually exclusive populations of DNA molecules. In this case, the fold enrichment in the sequential ChIP is within experimental error of the fold enrichment of the first ChIP, and this result is also independent of the order of the immunoprecipitations. Third, "partial co-occupancy" occurs when some DNA molecules have both factors, whereas other DNA molecules have only one of the two. There are two types of partial co-occupancy, which are distinguished by whether only one or both proteins can occupy the promoter in the absence of the other. In these two cases, the order of individual ChIPs often makes a difference, and partial co-occupancy is predicted to be observed in only one direction when one protein associates in a significantly sub-stoichiometric manner with respect to the other (see Supplemental Figure S1).

We define a measure of co-occupancy (C; in percent of complete co-occupancy which is defined as 100) as $C = 100(AB\text{-}A)/(A \times B\text{-}A)$, where A and B represent the fold enrichments for individual ChIPs and AB represents the fold enrichment for the sequential ChIP (see Supplemental Figure S1). The expressions (AB-A) and (A×B-A) represent the net increase (if any) in observed (AB) or predicted full (A×B) co-occupancy over the singly immunoprecipitated sample (A). If the fold enrichments of the sequential ChIP (AB) and corresponding single ChIP (A) are comparable, the proteins do not co-occupy the promoter and $C = 0$. Partial co-occupancy of two proteins on a promoter occurs when the fold enrichment of the sequential ChIP is significantly (at least 2-fold) higher than that of the corresponding single ChIP, but the C value is significantly below 100. We note that conclusions of complete co-occupancy (i.e., $C = 100$)

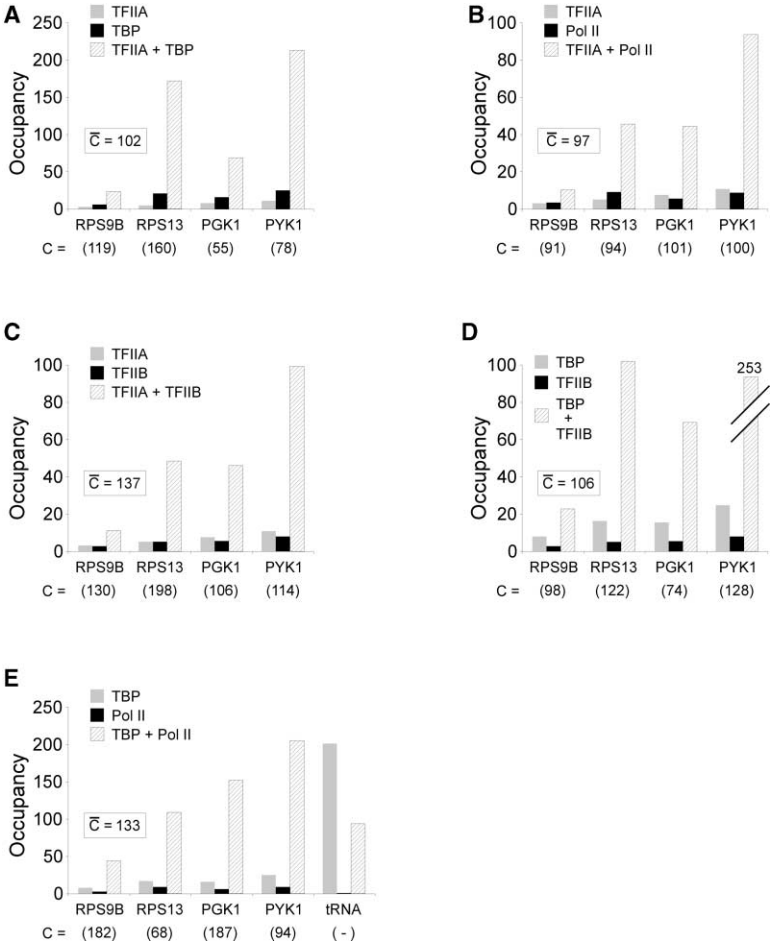

Figure 1. Complete Co-Occupancy of General Transcription Factors TBP, TFIIA, TFIIB, and Pol II In Vivo

Single occupancy and pairwise factor co-occupancy at the indicated promoters (fold enrichment over the *POL1* coding region control) are shown along with the calculated C values (see text). For each pair of factors tested, the mean co-occupancy for the four promoters is shown (overlined C in box). (A) TFIIA and TBP. (B) TFIIA and Pol II. (C) TFIIA and TFIIB. (D) TBP and TFIIB. (E) TBP and Pol II. C is undefined for the tRNA gene as Pol II occupancy is not detected.

are limited by experimental error and hence do not exclude the possibility of a small proportion of DNA molecules containing only one of the two relevant proteins.

To validate the sequential ChIP approach, we first examined co-occupancy of TBP, TFIIA, TFIIB, and Pol II at several promoters in normally growing cells (Figure 1). For all five pairwise combinations tested, the fold enrichments for the sequential ChIP experiments are comparable to the product of the individual fold enrichments (average C value = 97-137). Furthermore, in all cases tested, comparable C values are observed when the order of immunoprecipitations is reversed (Supplemental Figure S2). As expected, co-occupancy between TBP and Pol II was not observed at the Pol III-transcribed tRNA promoter (Figure 1E). These results demonstrate complete co-occupancy of the general transcription factors. As such, they provide direct evidence for the previous suggestion (Kuras and Struhl, 1999; Li et al., 1999) that intact preinitiation complexes are either present or absent at promoters and that partial preinitiation complexes do not exist to a significant extent in normally growing cells.

TAFs and Mot1 Partially Co-Occupy Promoters with TBP, but Their Association with Promoters Is Mutually Exclusive

Yeast promoters can be classified as either TFIID dependent or TFIID independent based on the requirement for TFIID-specific TAFs for transcription and on TAF:TBP occupancy ratios at promoters (Kuras et al., 2000; Li et al., 2000). We measured co-occupancy of TAFs with each other and with TBP at both TFIID-dependent (*RPS11B*, *RPL2B*, *RPS13*) and TFIID-independent (*ADH1*, *PYK1*, *PGK1*) genes. As expected from the fact that TAF12 and TAF6 are stoichiometric subunits of the TFIID complex, these TAFs show complete co-occupancy (average C value = 90-145) at all TAF-dependent and TAF-independent promoters tested (Figure 2A). On the other hand, co-occupancy of TBP with either TAF12 (Figure 2B; C = 18) or TAF6 (Figure 2C; average C value = 15) at TAF-independent genes is dramatically lower, consistent with the previously observed low TAF binding to these promoters (Kuras et al., 2000; Li et al., 2000). As expected, TBP co-occupancy with TAF12 (Figure 2B; average C value = 55) and TAF6 (Figure 2C; average C value = 38) at TFIID-dependent promoters is approximately 3-fold higher than those at their TFIID-independent counterparts. However, co-occupancy of TBP and TAFs at TFIID-dependent promoters appears to occur at a lower level than co-occupancy of TAF6 and TAF12, suggesting that TAFs are likely to be present in sub-stoichiometric amounts relative to TBP even at promoters where TFIID occupancy is relatively high.

We next analyzed co-occupancy of Mot1 and TBP to address whether Mot1 behaves like a general component of the transcriptional machinery or a sub-stoichio-

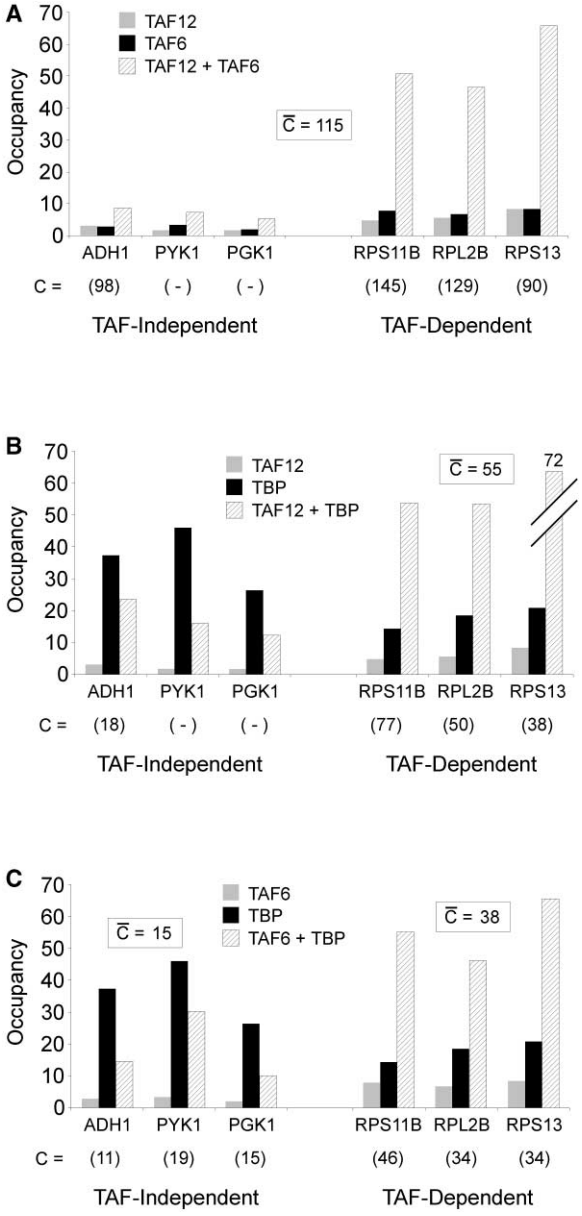

Figure 2. Sub-Stoichiometric Co-Occupancy of TBP with TAFs

Single occupancy and pairwise factor co-occupancy at TAF-independent (*ADH1, PYK1, PGK1*) and TAF-dependent (*RPS11B, RPL2B, RPS13*) promoters are shown along with the calculated C values (dashes indicate that TAF occupancy is too low to permit a meaningful value). Where appropriate, mean promoter co-occupancies (overlined C in box) across promoters that are TAF independent, TAF dependent, or both are shown. (A) TAF6 and TAF12. (B) TAF12 and TBP. (C) TAF6 and TBP.

metric TAF (Figure 3A). In all cases, the sequential ChIP sample shows a 3- to 4-fold increase in enrichment over the sample individually immunoprecipitated with Mot1, indicating that Mot1 and TBP can co-occupy all four promoters. However, the average C value is only 10, indicating that Mot1 and TBP co-occupy active promoters far less often than TBP and Pol II. When the order of immunoprecipitation is reversed, the fold enrichment in the sequential ChIP sample is comparable to the sample individually immunoprecipitated with TBP (Supplemental Figure S3). As discussed above (see also Supplemental Figure S1), the unidirectional nature of the Mot1-TBP co-occupancy indicates that most promoter-bound TBP molecules are not associated with Mot1, a result in accord with previous suggestions (Geisberg et al., 2002).

Although Mot1 and TAFs each partially co-occupy promoters with TBP, sequential ChIP analysis indicates that Mot1 does not co-occupy promoters with either TAF12 (Figure 3B) or TAF6 (Figure 3C). This observation, coupled with the observed substoichiometric association of Mot1 and TAFs with promoter-bound TBP, indicates that promoter binding by Mot1 and TFIID occurs on mutually exclusive DNA molecules in vivo. This view is consistent with biochemical evidence that Mot1 and TAFs are found in mutually exclusive TBP complexes (Poon et al., 1994) and that Mot1 does not affect TFIID-dependent transcription in vitro (Chicca et al., 1998).

Mot1 Does Not Co-Occupy Promoters with TFIIB, TFIIA, and Pol II in Normally Growing Cells

To address the key question of whether Mot1 is a component of a transcriptionally active form of TBP or is in equilibrium with active forms of TBP, we used sequential ChIP to determine whether Mot1 can co-occupy promoters with TFIIB, TFIIA, and Pol II (Figure 3). In cells grown in standard medium at 30°C, the fold enrichments for any of the sequential ChIP experiments involving Mot1 at the *RPS9B*, *RPS13*, *PYK1*, and *PGK1* promoters are indistinguishable from the fold enrichments of Mot1 alone (factor of 1.1 for TFIIA, 1.1 for TFIIB, and 1.5 for Pol II when averaged over the four promoters tested; as mentioned above, parallel analyses of TBP resulted in 3- to 4-fold enrichment). Similar results were obtained when these sequential ChIP experiments were performed in the reverse order (i.e., immunoprecipitation of TFIIA, TFIIB, and Pol II first, followed by immunoprecipitation of Mot1; Supplemental Figure S3). Thus, Mot1 does not co-occupy promoters with TFIIB, TFIIA, or Pol II in normally growing cells, and hence is not a part of active transcription complexes under these conditions. However, the presence of Mot1 at promoters in a transcription-dependent manner indicates that Mot1-TBP complexes do not simply associate with promoter sequences, but rather are in dynamic equilibrium with transcriptionally active forms of TBP.

Mot1 Can Co-Occupy Promoters with TFIIB and Elongation-Competent Pol II, but Not TFIIA, under Conditions of Heat Shock Stress

We performed similar sequential ChIP experiments involving Mot1, TFIIB, TFIIA, and Pol II in cells subjected to heat shock, which induces a large number of stress genes such as *HSP104*, *SSA4*, *SSA3*, and *CTT1*. In contrast to the situation in normally growing cells, we observe significant co-occupancy of Mot1 with both TFIIB (Figure 4A) and Pol II (Figure 4B) in heat-shocked cells. Strikingly, such co-occupancy is not restricted to transcriptionally induced genes, because promoters not regulated by these stress conditions (*PGK1* and *PYK1*) behave in a comparable manner. The co-occupancy of Mot1 with TFIIB (average C value = 33) and Pol II (average C value = 38) is incomplete, because the average

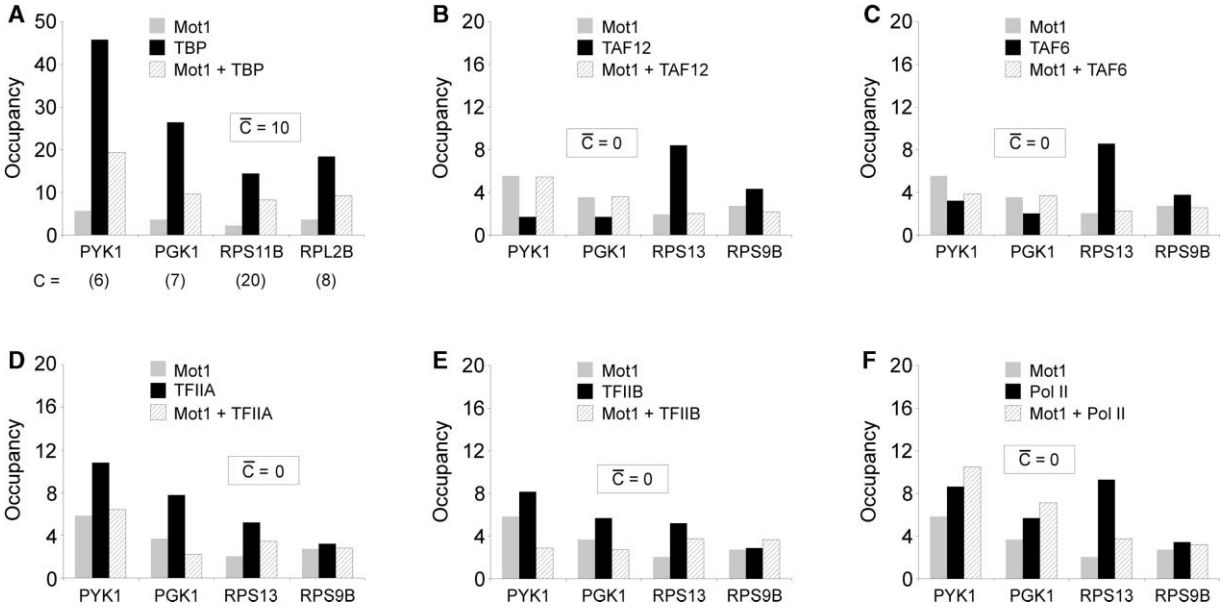

Figure 3. Mot1 Co-Occupies with TBP, but Not with TAF6, TAF12, TFIIA, TFIIB, or Pol II Under Normal Growth Conditions

Single and pairwise factor co-occupancy at the indicated promoters in cells grown at 30°C. C values are calculated only for the combination of Mot1 and TBP, because the fold enrichments of Mot1 and other pairwise combinations involving Mot1 are within experimental error (C values are effectively 0). (A) Mot1 and TBP. (B) Mot1 and TAF12. (C) Mot1 and TAF6. (D) Mot1 and TFIIA. (E) Mot1 and TFIIB. (F) Mot1 and Pol II.

C values are lower than those observed for general transcription factors and are more in line with TAF-TBP and Mot1-TBP co-occupancies. Partial co-occupancy of Mot1 with these general factors is expected, because Mot1 is substoichiometric with respect to TBP, and hence is unlikely to present at all preinitiation complexes. These results suggest that Mot1-TBP complexes can be part of preinitiation complexes under conditions of environmental stress.

Phosphorylation of the C-terminal domain (CTD) of Pol II at serine 5 (CTD-serine 5-P) occurs after preinitiation complex formation, and it is a distinguishing feature of the elongation-competent transcription machinery (Komarnitsky et al., 2000; Schroeder et al., 2000). To determine whether Mot1 can associate with transcriptionally competent Pol II, we performed sequential ChIP experiments using antibodies against CTD-serine 5-P (Figure 4C). Under conditions of heat shock, Mot1 co-occupies both heat-shock regulated (*HSP104*, *SSA4*, *SSA3*, and *CTT1*) and nonregulated (*PGK1*, *PYK1*) promoters with the CTD-serine 5-P form of Pol II. Co-occupancy of Mot1 and phospho-Pol II (average C value = 21) roughly comparable (perhaps slightly lower) than that observed for Mot1-TFIIB and Mot1-Pol II (non-phosphorylated form). Finally, co-occupancy between Mot1 and the CTD-serine 5-P form of Pol II is not observed in the absence of heat shock stress. These observations indicate that Mot1 associates with a form of the Pol II machinery that arises after preinitiation complex formation and is very likely to be transcriptionally active.

Although Mot1 can co-occupy promoters with TFIIB and Pol II under conditions of heat shock stress, Mot1 does not co-occupy promoters with TFIIA under these same conditions (Figure 4D). This result is significant, because analysis of the identical samples indicates that TFIIA fully co-occupies promoters with TFIIB and Pol II under both normal and stress conditions (data not shown), and it co-occupies promoters with TAFs (data not shown). The lack of co-occupancy by Mot1 and TFIIA in vivo is in accord with their functional antagonism in vitro (Auble and Hahn, 1993; Auble et al., 1994; Chicca et al., 1998), which presumably is due to competitive binding to the solvent-exposed surface of TBP (Cang et al., 1999). The fact that both Mot1 and TFIIA associate with promoters containing active transcription complexes, but are not present at those promoters concurrently, implies that stress-inducible transcription involves distinct Mot1-TBP- and TFIIA-TBP-containing complexes. Furthermore, the results suggest that transcriptional complexes can be active in the absence of TFIIA, and that yeast cells have an alternate form of the preinitiation complex in which Mot1 replaces TFIIA.

Co-occupancy of Mot1 and Pol II Can Occur during Copper and Hyperosmotic Stress

To examine if Mot1 co-occupancy with Pol II is unique to heat shock or is a more general stress phenomenon, we subjected yeast cells to high copper concentrations (Figure 5A) and hyperosmotic stress (Figure 5B). These conditions result, respectively, in recruitment of Mot1 and Pol II to copper-regulated (*CUP1*, *CRS5*) or salt-inducible (*STL1*, *GPD1*) promoters. Mot1 and Pol II co-occupy the copper regulated (average C value = 33) and osmotically induced (C = 16) promoters, but only under inducing conditions. Co-occupancy of Mot1 and Pol II is partial, and occurs at a level roughly comparable to that observed for heat shock-regulated promoters. In the presence of high copper concentrations, Mot1

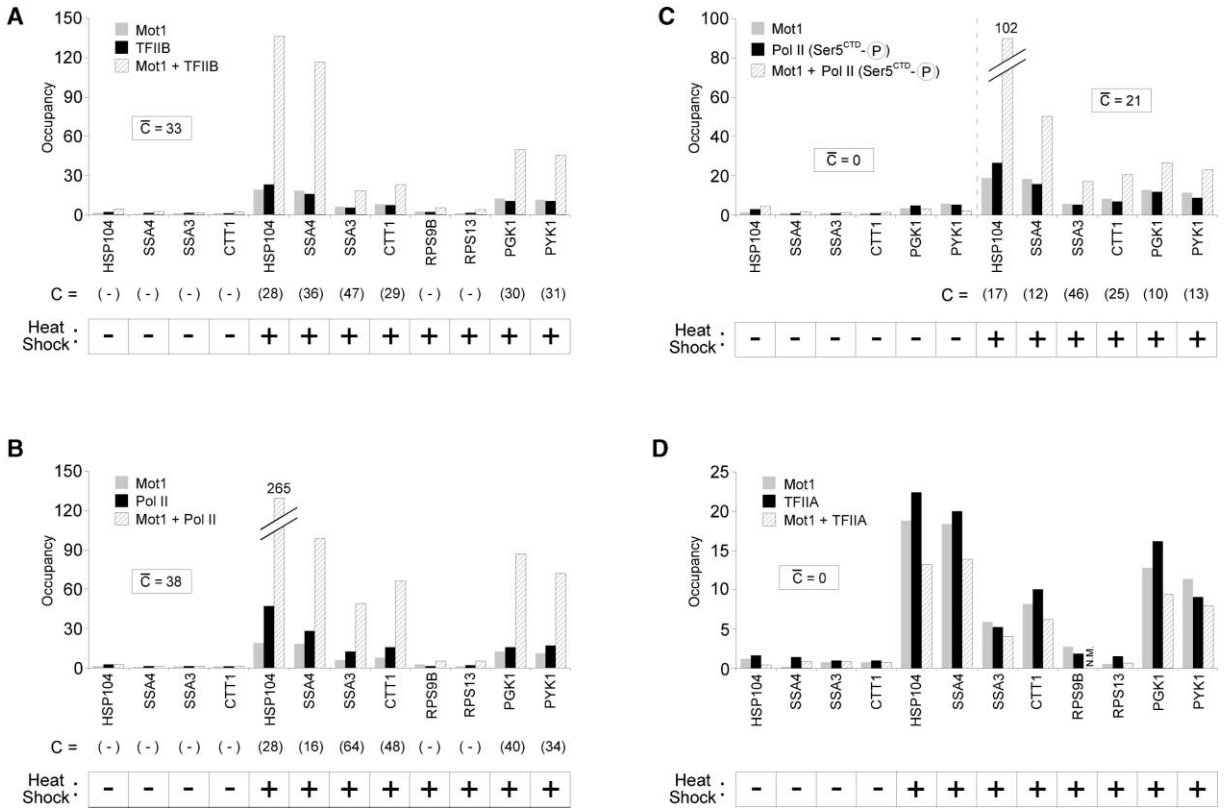

Figure 4. Mot1 Co-Occupies Promoters with TFIIB and Pol II, but Not TFIIA, Under Conditions of Heat Shock Stress

Single and pairwise factor co-occupancy at the indicated promoters in cells that were (+) or were not (-) heat shocked for 15 min at 39°C. C values for individual promoters are shown for heat-shocked cells. (A) Mot1 and TFIIB. (B) Mot1 and Pol II. (C) Mot1 and Pol II-CTD-serine 5-P. Single factor occupancy at the *PGK1* and *PYK1* promoters at 30°C is significant, but the fold enrichments of Mot1 and the pairwise combinations are within experimental error (C = 0). (D) Mot1 and TFIIA.

and Pol II co-occupy the nonregulated *PGK1* promoter, mirroring the behavior of this promoter under heat shock stress. Thus, copper stress alters the functional properties of Mot1 in a manner that is not restricted to copper-inducible genes. We cannot determine whether Mot1 and Pol II co-occupy nonregulated promoters under hyperosmotic stress, because this condition results in a general reduction in protein association (Proft and Struhl, unpublished data). Taken together, these observations suggest that general stress response results in an increased proportion of transcriptionally active Mot1 complexes on both regulated and unregulated promoters.

Indistinguishable Kinetics of Mot1 and TFIIA Association upon Transcription Induction

The mutual exclusivity of Mot1 and TFIIA at heat shock-inducible promoters could reflect binding of Mot1-TBP and TFIIA-TBP complexes in a specific temporal order (e.g., Mot1-TBP binding first, later to be displaced by other TBP- and TFIIA-containing complexes). To address this possibility, we examined Mot1 and TFIIA binding to three heat-shock promoters (*HSP104*, *SSA4*, and *CTT1*) at 1 min intervals following a heat shock. As shown in Figure 6, Mot1, TFIIA, TBP, and Pol II bind in a kinetically indistinguishable fashion, with full occupancy achieved between 3–5 min after heat shock, depending on promoter. Likewise, the dissociation rates of the four factors at a ribosomal protein promoter (*RPL41B*) were very similar to one another. The absence of clear, stepwise ordered binding of TBP complexes suggests TFIIA- and Mot1-containing TBP complexes are recruited independently of one another, and are in dynamic equilibrium at the promoter.

Discussion

Sequential ChIP as a Method for Analyzing Transcriptional Regulatory Mechanisms In Vivo

Sequential ChIP has been used to address whether two different proteins co-occupy a given genomic region (Scully et al., 2000; Chaya et al., 2001; Proft and Struhl, 2002; Soutoglou and Talianidis, 2002). Here, we provide a theoretical basis for interpreting sequential ChIP experiments in a quantitative manner (Supplemental Figure S1), and we demonstrate that sequential ChIP is a valid approach for analyzing the components of preinitiation complexes in vivo. For all cases where detailed biochemical and structural information is available, our sequential ChIP results are in excellent accord with expectations. For example, it is widely assumed that basic components of the preinitiation complex (e.g., TBP, TFIIB, TFIIA, Pol II) completely co-occupy the promoter, and indeed we show that the fold enrichments in the

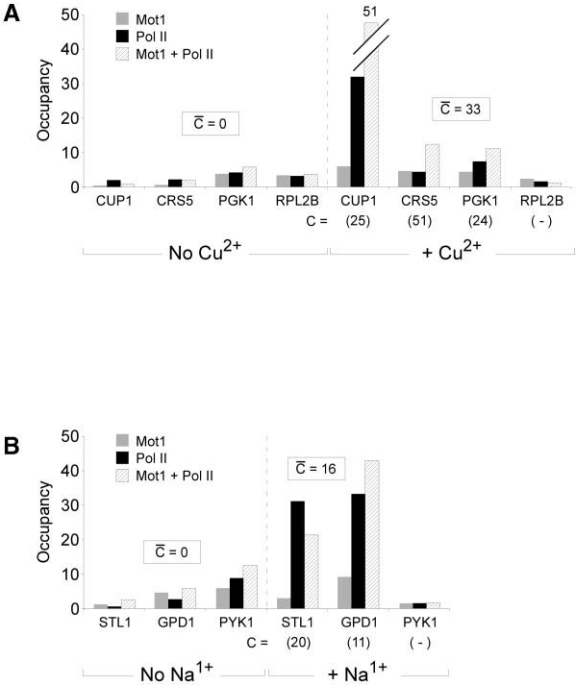

Figure 5. Mot1 and Pol II Co-Occupancy Promoters in Response to Copper and Hyperosmotic Stress

Single and pairwise factor co-occupancy at the indicated promoters in normal or stressed cells, with C values indicated where appropriate. (A) Cells were treated with 500 μM $CuSO_4$ for 10 min. No C value is given for *RPL2B* because copper stress reduces Mot1 and Pol II occupancy to near background levels. (B) Cells were treated with 0.4 M NaCl for 5 min. No C value is given for *PYK1* because copper stress reduces Mot1 and Pol II occupancy to near background levels.

sequential ChIP experiment are comparable to the product of the fold enrichments of the individual ChIP experiments. Similarly, we observe complete co-occupancy of TAF6 and TAF12, as expected from the fact that these proteins are obligate components of the TFIID complex. In contrast, our observation of partial co-occupancy of TBP with TAFs or Mot1 is consistent with the strong suggestions that TAFs (Kuras et al., 2000; Li et al., 2000) and Mot1 (Geisberg et al., 2002) are substoichiometric with respect to TBP in vivo. Lastly, biochemical experiments strongly suggest that TAFs and Mot1 associate with TBP in a mutually exclusive manner (Poon et al., 1994; Chicca et al., 1998), and our sequential ChIP results indicate no co-occupancy of Mot1 and TAFs in vivo.

Distinct Forms of Mot1 at Transcriptionally Active Promoters

Mot1 associates with transcriptionally active promoters, and it can directly activate or inhibit transcription in an ATPase-dependent manner (Dasgupta et al., 2002; Geisberg et al., 2002). However, Mot1 also evicts TBP from DNA in an ATP-dependent manner in vitro (Auble and Hahn, 1993; Auble et al., 1994) and in vivo (Li et al., 1999; Geisberg et al., 2002). Thus, a critical and heretofore unresolved issue is whether the Mot1-TBP complex is a TAF-independent form of transcriptionally active form of TBP or instead is an inactive form that is in dynamic equilibrium with active TBP forms (e.g., TFIID and free TBP). Using sequential ChIP, we show here that promoter-associated Mot1 exists both in transcriptionally active and inactive forms, depending on the environmental conditions.

In normally growing cells, Mot1 does not co-occupy promoters with Pol II, TFIIB, TFIIA, or TAFs. Thus, under normal growth conditions, promoter-bound Mot1 (and presumably the Mot1-TBP complex) is in dynamic equilibrium with active transcription complexes. Although Mot1-TBP complexes under these circumstances are transcriptionally inactive, it is unclear whether they block the association of active TBP complexes at the promoter. TBP association at nearly all Pol II promoters is well below full occupancy (Kuras and Struhl, 1999), so competition between Mot1-TBP and active TBP complexes for the TATA element may not be a physiologically relevant issue. However, if Mot1 is drawn to active promoters simply because there are high levels of TBP, much like an enzyme associates with its substrate, Mot1 would presumably reduce the concentration of active TBP at the promoter and hence inhibit transcription. In any event, our results clearly show that Mot1-TBP is not a significant TAF-independent form of transcriptionally active TBP in normal cells.

In striking contrast to the situation in normal cells, Mot1 co-occupies promoters with Pol II and TFIIB under conditions of heat shock and other forms of cellular stress. Importantly, Mot1 co-occupancy with Pol II and TFIIB is observed both at stress-induced and unaffected promoters, indicating that cellular stress alters the functional properties of Mot1 in a manner independent of transcriptional induction per se. Under these stress conditions, Mot1-TBP complexes are clearly part of preinitiation complexes containing Pol II. Furthermore, in heat-shocked cells, Mot1 co-occupies promoters with Pol II whose CTD is phosphorylated at serine 5, indicating that Mot1 is part of a transcription complex that arises after preinitiation complex formation. As Pol II phosphorylated at CTD-serine 5 is associated with elongation, it is very likely (and we will subsequently assume) that complexes containing Mot1, TFIIB, and this serine 5-phosphorylated form of Pol II are transcriptionally competent. However, we cannot exclude the formal possibility Mot1 might inhibit transcription in a hypothetical step after CTD phosphorylation and prior to elongation.

Mot1 Can Replace TFIIA in Functional Preinitiation Complexes In Vivo

Under conditions of cellular stress, the preinitiation complex (defined here as TBP, TFIIB, and Pol II) co-occupies promoters with Mot1 or with TFIIA, but association of Mot1 and TFIIA occurs on mutually exclusive DNA molecules. This strongly suggests that stressed cells have distinct Mot1- and TFIIA-containing preinitiation complexes that contribute to transcriptional activity. The existence of Mot1-containing preinitiation complexes can explain why TFIIA-depleted cells show a general but rather modest (3-fold) reduction in transcription (Kang et al., 1995; Chou et al., 1999; Liu et al., 1999). As TFIIA is essential for cell viability, TFIIA-depleted cells are likely to be undergoing stress, and hence are likely to contain the active form of Mot1.

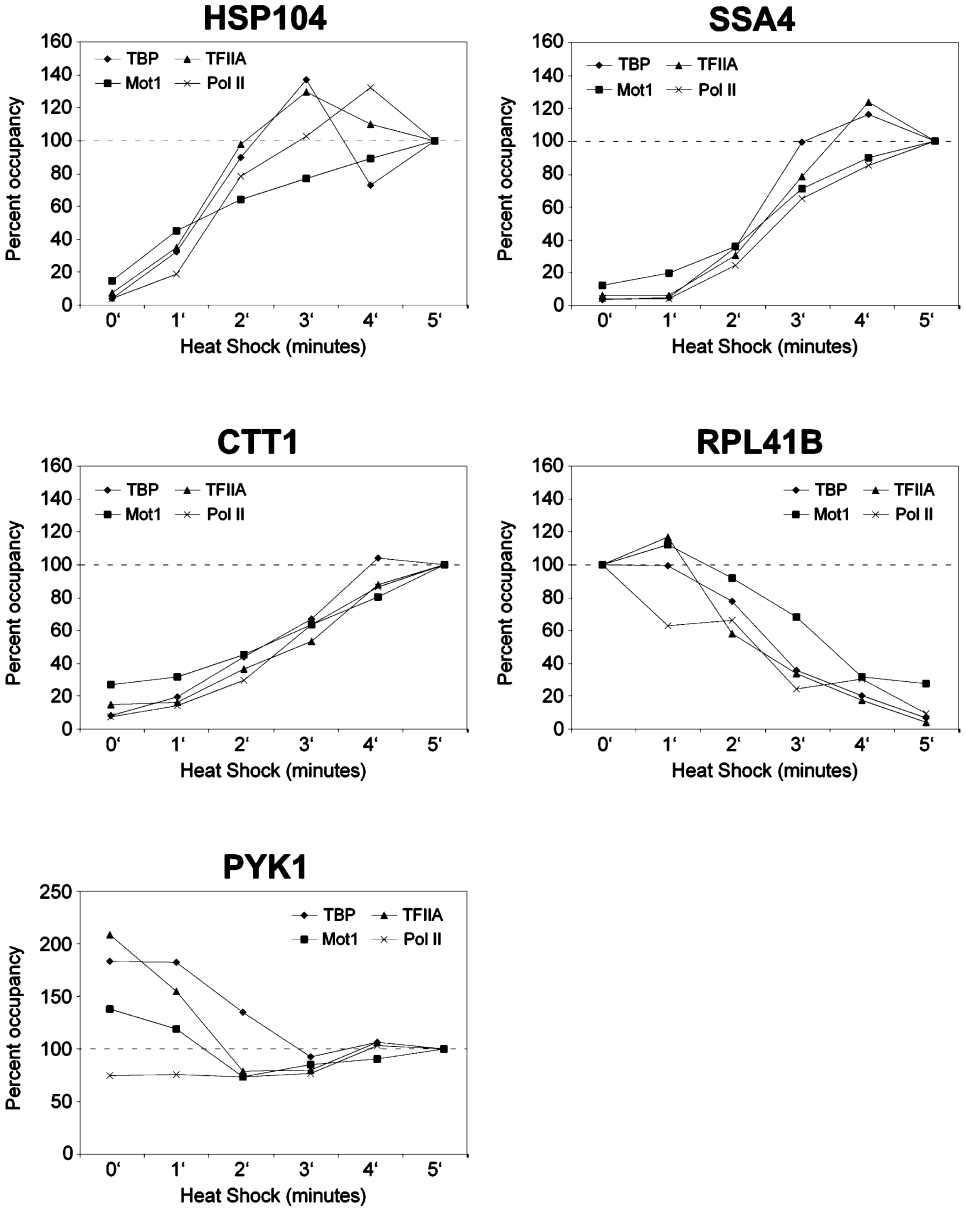

Figure 6. Kinetics of Mot1, TBP, TFIIA, and Pol II Occupancy upon Heat Shock Stress

Occupancy of the indicated factors in cells that were heat-shocked at 39°C for the indicated time. Occupancy values were normalized relative to the 5 min time point for genes induced or minimally affected by heat shock (*HSP104*, *SSA4*, *CTT1*, *PYK1*) and to the 0 min time point for *RPL41B*, whose transcription drops upon heat shock.

Our results also suggest that Mot1 not only blocks TFIIA function in transcription, but that it can also replace TFIIA in functional preinitiation complexes. TFIIA and Mot1 each contact DNA, so it is likely that they help stabilize TBP association with the promoter. Interestingly, both Mot1 (Gumbs et al., 2003) and TFIIA (Stewart and Stargell, 2001) alter the DNA binding specificity of TBP in distinct manners, suggesting that the Mot1- and TFIIA-containing preinitiation complexes will have different core promoter specificities. In this regard, Mot1-dependent effects on transcription can depend on the functional quality and DNA sequence in the vicinity of the TATA region (Collart, 1996; Geisberg et al., 2002). It is also possible that the Mot1- and TFIIA-containing preinitiation complexes might respond differentially to activator or repressor proteins.

Mot1-Dependent Effects on Transcription

Our results strongly suggest that the Mot1-TBP complex is a TAF-independent form of transcriptionally active TBP in stressed cells. However, it is difficult to assess the relative levels and individual contributions of Mot1-TBP, TFIID, and free TBP to the overall transcriptional activities at promoters in wild-type cells. On average, TFIIA-dependent transcription accounts for approximately two-thirds of the total level of transcription (Kang

et al., 1995; Chou et al., 1999; Liu et al., 1999), and the level of Mot1-TBP co-occupancy (average C value = 10) is relatively low. For these reasons, we suspect that, in general, Mot1-containing preinitiation complexes make a modest contribution to overall transcription levels at most promoters. Interestingly, transcriptional profiling experiments indicate that stress-response genes are significantly overrepresented among Mot1-regulated genes (Andrau et al., 2002; Dasgupta et al., 2002; Geisberg et al., 2002), and Mot1-regulated genes have unusually high Mot1:TBP occupancy ratios (Dasgupta et al., 2002; Geisberg et al., 2002). Thus, it is possible that Mot1-containing preinitiation complexes might make a more significant impact on overall transcription at selected genes.

It has been presumed that Mot1 performs the same TBP-dependent function(s) at all promoters, but that promoter-specific implementation of this function(s) accounts for the positive and/or negative transcriptional effects observed in vivo. However, we show here that Mot1 is not functionally equivalent under all conditions. Thus, Mot1-stimulatory and Mot1-inhibitory effects on transcription might be due to differential association of two functionally distinct (active and inactive) forms of Mot1-TBP on specific promoters. It is important to note that Mot1-regulated genes are defined by comparing healthy wild-type cells (where Mot1 is not transcriptionally competent) to *mot1* mutant cells that are presumably under physiological stress due to very low levels of Mot1 (which is likely to be transcriptionally active). As stress in *mot1* cells is likely to an inseparable byproduct of Mot1 inactivation, the definition of Mot1-regulated genes incorporates conditions of stress (in which active forms of Mot1 are present) in addition to the effects attributed to Mot1 inactivation.

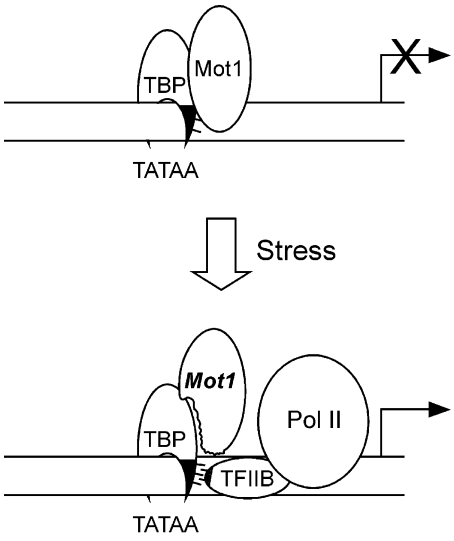

Figure 7. Models for Stress-Dependent Conversion of Transcriptionally Inactive Promoter-Bound Mot1-TBP Complexes to an Active Form

Under normal growth, Mot1 cannot form active Pol II-containing complexes either because it masks the TFIIB-interaction surface of TBP (black shaded region with dashes depicting protein-protein contacts) or competes with TFIIB for a common surface of DNA. Cellular stress results in a chemical modification (e.g., phosphorylation) and/or conformational change in Mot1, thereby weakening the interaction between Mot1 and the TFIIB binding surface of TBP or between Mot1 and the DNA surface normally contacted by TFIIB. This in turn allows for the association of TFIIB and other required components of the Pol II transcription machinery, ultimately resulting in transcription. Related models invoking a stress-induced change in TBP or TFIIB are possible, although such modifications would have to be specific for Mot1-related functions of TBP or TFIIB.

How Does Cellular Stress Alter Mot1 Function?

There are two basic models to explain how cellular stress converts the Mot1-TBP complex from a transcriptionally inactive form to a transcriptionally active form. In one model, stress induces a covalent modification (e.g., phosphorylation, ubiquitination, proteolytic cleavage) or conformational change in Mot1. Mot1 is a large protein that contains many potential phosphorylation and glycosylation sites, but it is unknown if these are actually used. Alternatively, stress modifies TBP, TFIIB, or (less likely) some other component of the basic transcription machinery that functionally interacts with Mot1. Although Mot1 function can be altered by different forms of stress, it is unclear whether there are common or multiple inducing signals and whether the signals are comparable to those governing the general stress response (Gasch et al., 2000; Causton et al., 2001).

Whatever the molecular modification induced by stress, the key functional distinction between transcriptionally active and inactive forms is whether or not Mot1 is part of a preinitiation complex. TBP interacts directly with Mot1 and TFIIB, and there is no evidence for Mot1 interactions with other general factors. Thus, an attractive hypothesis is that the two Mot1-TBP forms differ by their ability to interact with TFIIB (Figure 7). For example, Mot1 might interact with and mask the TFIIB-interaction surface of TBP, thereby blocking TFIIB (and subsequent factor) assembly into an active preinitiation complex. Cellular stress would cause a modification that weakens or eliminates the interaction between Mot1 and the TFIIB-interaction surface of TBP, thereby permitting TFIIB association and preinitiation complex formation. Alternatively, Mot1 and TFIIB might compete for a common DNA binding surface that is necessary for TFIIB to form a stable TBP-TFIIB-TATA complex. Cellular stress might weaken the Mot1-DNA interaction, thereby resulting in an increase in the formation of active preinitiation complexes. We slightly disfavor this model because the efficiency of Mot1 crosslinking to promoters is comparable in normal and stressed cells. Both models are consistent with the observation in vitro that Mot1 does not efficiently bind or disrupt (in the presence of ATP) TFIIB-TBP-TATA complexes (Auble and Hahn, 1993). The simplest version of both models would involve a modification of Mot1, because modifications of TBP (or perhaps TFIIB) would have to selectively affect the Mot1 interaction and not significantly disrupt other functions of TBP and TFIIB. Although the precise mechanistic details remain to be elucidated, our results demonstrate that cellular stress alters the transcriptional properties of Mot1-TBP complexes, and that the Mot1-TBP complex can be part of a distinct and functional form of the Pol II transcription machinery that might differentially affect gene expression.

Experimental Procedures

Yeast Strain Growth

Saccharomyces cerevisiae strain LK25 expresses a TBP derivative tagged at the N terminus with three copies of the HA1 epitope and a Mot1 derivative containing nine copies of the Myc epitope at the C terminus (Geisberg et al., 2002). The strain was grown, unless otherwise indicated, at 30°C in casamino acids medium containing 2% dextrose to an optical density at 600 nm of 0.6. For heat shock stress, cells were transferred to a 39°C shaking water bath for 15 min, except for the kinetic experiment (Figure 6), where cells were incubated for shorter times. Copper stress was achieved by growing cells in SD medium containing all essential amino acids, nucleotides, and 2% dextrose and adding 500 μM $CuSO_4$ for 10 min. For hyperosmotic stress, cells grown in casamino acids containing 2% dextrose at 30°C were treated with 0.4 M NaCl for 5 min.

Chromatin Immunoprecipitation

Chromatin isolation and single chromatin immunoprecipitations in yeast cells were performed essentially as described previously (Kuras and Struhl, 1999; Komarnitsky et al., 2000; Geisberg et al., 2002) using antibodies against the HA epitope (F-7; Santa Cruz Biotech), Myc epitope (06-549; Upstate Biotechnology), TFIIA and TFIIB (Kuras et al., 2000), TAF6 and TAF12 (kindly provided by Michael Green), non-phosphorylated C-terminal domain of Pol II (8WG16; Covance), CTD phosphorylated Pol II (Ser-5 phosphorylated from Invitrogen; also kindly provided by Michael Keogh and Steve Buratowski).

Sequential chromatin immunoprecipitation was performed with minor modifications of a method described previously (Proft and Struhl, 2002). Crosslinked chromatin from approximately 0.5 to 1 × 10^9 cells in 1 ml of FA buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 2 mM phenyl methyl sulfonyl fluoride) was precipitated with 5–20 μl of the first antibody and 50 μl of protein A-sepharose (50% volume:volume in Tris-buffered saline) for 90 min at room temperature. Bound complexes were washed (1 ml solution for 5 min at room temperature) either six times with FA buffer (for F7 or 9E10 antibodies) or (for all other antibodies) three times with FA buffer, once with FA buffer containing 0.5 M NaCl, once with 10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate, and finally once with TE (pH 8.0). For immunoprecipitations involving the F7 or 9E10 antibodies, complexes were eluted with 100 μl of TBS buffer containing 1 mg/ml HA-1 or Myc peptide. For all other immunoprecipitations, complexes were eluted in 100 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% SDS, and heating to 65°C for 10 min.

The eluates from the first immunoprecipitation were adjusted to 1 ml FA buffer containing 25 μg/ml phage λ DNA, 5 mg/ml Bovine Serum Albumen (Fraction V; Sigma), and 50 μg/ml *E. coli* tRNA. The resulting mixture was then immunoprecipitated with 5-20 μl of the second antibody and 50 μl protein A-Sepharose beads (50% volume:volume in Tris buffered saline) for 90 min at room temperature. Subsequent washes and elution steps were identical to those described above. Comparable results are obtained with different methods of elution (peptide or heating), type of antibody (monoclonal or polyclonal), or nature of the protein (epitope-tagged or untagged).

To ensure consistency, sequential ChIP experiments were performed in parallel. Specifically, a given chromatin sample was immunoprecipitated with multiple antibodies in parallel, and the resulting eluates (i.e., individual ChIPs) were immunoprecipitated with multiple antibodies in parallel. All experiments have been repeated a minimum of three independent times with very reproducible results, eliminating the possibility that a second immunoprecipitation might have failed for technical reasons.

Quantitative real-time PCR with gene-specific oligonucleotides were performed as described (Geisberg et al., 2002) on Applied Biosystems 7700 and 7000 detectors. For any given sample, input-normalized occupancy units (for each primer pair) are expressed as fold occupancy over an internal fragment of the *POL1* gene (Geisberg et al., 2001). Occupancy values represent an average of at least three independent experiments and have an error of approximately ± 25%; very low occupancy values may have greater errors. In comparing fold enrichments following sequential immunoprecipitations to the corresponding single immunoprecipitation, it is important to note that experimental errors are associated independently with each measurement. For this reason, we consider 2-fold differences between sequential and individual immunoprecipitation to be significant and indicative of co-occupancy. Differences that are less than 2-fold are within experimental error and are assumed to indicate no detectable co-occupancy. Co-occupancy at a given promoter cannot be determined if either individual immunoprecipitation results in a <2-fold enrichment.

Genome-Wide Location of Mot1

DNA from immunoprecipitated Mot1 complexes was amplified in the presence of 0.3 mM amino-allyl-dUTP (Sigma) and labeled with Cy5 fluorescent dye (Amersham Biosciences) as described (Iyer et al., 2001). Control samples consisting of input DNA were amplified in a similar fashion but were labeled with the Cy3 dye. A mixture of immunoprecipitated and input DNA was then hybridized overnight to polylysine-coated glass slides spotted in duplicate with a set of ~6,500 yeast intergenic sequences generated by PCR (Moqtaderi and Struhl, 2004). Slides were washed, scanned on an Axon scanner, and the resulting data and images were analyzed and refined using Axon GenePix 4.0 software. The ratio of Cy5 to Cy3 was calculated for each spot, and the median ratio normalized to 1 in order to permit cross-slide comparison. Median occupancy values from three experiments representing independent cell populations (six data sets) were calculated for every intergenic region. Occupancy values more than 2 were above the median and were scored as positives. Statistical significance between Mot1 occupancy and transcriptional frequency (Holstege et al., 1998); http://web.wi.mit.edu/young/expression/halflife.html) was measured by a non-parametric median test (http://www.fon.hum.uva.nl/Service/Statistics/Median_Test.html) because neither variable necessarily follows a normal distribution. Gene Ontology (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermMapper) was used to test whether a group of biologically related genes is overrepresented in the genes scored positive for Mot1 occupancy.

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