Cooperative action of NC2 and Mot1p to regulate TATA-binding protein function across the genome

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Promoter recognition by TATA-binding protein (TBP) is an essential step in the initiation of RNA polymerase II (pol II) mediated transcription. Genetic and biochemical studies in yeast have shown that Mot1p and NC2 play important roles in inhibiting TBP activity. To understand how TBP activity is regulated in a genome-wide manner, we profiled the binding of TBP, NC2, Mot1p, TFIID, SAGA, and pol II across the yeast genome using chromatin immunoprecipitation (ChIP)–chip for cells in exponential growth and during reprogramming of transcription. We find that TBP, NC2, and Mot1p colocalize at transcriptionally active pol II core promoters. Relative binding of NC2/H9251 and Mot1p is higher at TATA promoters, whereas NC2/H9252 has a preference for TATA-less promoters. In line with the ChIP–chip data, we isolated a stable TBP–NC2–Mot1p–DNA complex from chromatin extracts. ATP hydrolysis releases NC2 and DNA from the Mot1p–TBP complex. In vivo experiments indicate that promoter dissociation of TBP and NC2 is highly dynamic, which is dependent on Mot1p function. Based on these results, we propose that NC2 and Mot1p cooperate to dynamically restrict TBP activity on transcribed promoters.

[Keywords: TATA-box-binding protein; NC2; Mot1; TFIID; SAGA; genome-wide location analysis]

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NC2 and mot1 mutants identified genes that were repressed, but also genes that were positively regulated by these factors [Geisberg et al. 2001; Andrau et al. 2002, Cang and Prelich 2002, Dasgupta et al. 2002]. Chromatin immunoprecipitation [ChIP] analysis indicated that NC2 and Mot1p can localize to actively transcribed genes [Andrau et al. 2002, Creton et al. 2002, Geisberg et al. 2002]. In addition, ChIP–reChIP experiments indicated that Mot1p, TFIIH, and RNA pol II can co-occupy heat stress-induced promoters [Geisberg and Struhl 2004]. Also, genetic interactions between mot1 mutants with spT8 and spT3 deletion strains suggest that there is a functional link between transcriptional activators like SAGA and Mot1p [Collart 1996; Madison and Winston 1997, van Oevelen et al. 2005]. A recent report indicates that NC2 can also stimulate PIC complex formation at selective promoters [Masson et al. 2008].

It is clear that Mot1p, NC2, SAGA, and TFIID can regulate TBP distribution and activity. A detailed view of how these factors cooperate is lacking, however. We addressed this by profiling the genome-wide localization of TBP, NC2, and Mot1p for yeast cells in exponential growth and during transcriptional reprogramming in a shift from high to low glucose. To examine the interplay with TFIIH, SAGA, and transcription, the binding profiles of Taf1p (TFIID), Spt20p (SAGA), and Rpb3p (pol II) were also determined. Our data indicate that there is substantial overlap between the TBP, NC2, and Mot1p binding profiles. The binding of NC2 and Mot1p also correlates with SAGA and TFIIID occupancy. Furthermore, NC2 and Mot1p binding show a strong correlation with active transcription. During the low glucose shift, the NC2α and NC2β subunits are differentially localized. We isolated a stable NC2–Mot1p–TBP–DNA complex, which is disrupted upon ATP hydrolysis. Based on these results, we propose that Mot1p and NC2 act in a cooperative mechanism to regulate the transcriptional output of active genes.

Results

Genomic binding profiles of TBP, NC2α, NC2β, and Mot1p correlate with active transcription

In trying to understand the functional interaction between NC2 and Mot1p, we profiled their genomic binding across the yeast genome and compared these with TBP binding. In addition, we examined the genomic distribution of Taf1p and Spt20p. Taf1p is the largest subunit of the TFIID complex, which consists of TBP and 13–14 evolutionarily conserved TBP-associated factors [TAFs] [Sanders and Weil 2000]. Both TAF-dependent and TAF-independent forms of TBP have been detected on active promoters [Kuras et al. 2000, Li et al. 2000]. TFIID shares some of its TAF subunits with SAGA/SILK coactivator complexes [Grant et al. 1998]. Spt20p is a core subunit of this multifunctional histone acetyl transferase complex, which coactivates transcription through different mechanisms [Timmers and Tora 2005, Daniel and Grant 2007]. To correlate the genomic distribution of these factors to the transcription state of genes we also included the Rpb3p subunit of pol II. Two biological replicates were processed for ChIP analysis. Cross-linked protein–DNA complexes were recovered via a biotin tag engineered at the N- or C-terminal end of the proteins [van Werven and Timmers 2006]. High-density oligonucleotide arrays covering the whole Saccharomyces cerevisiae genome and a T7 RNA polymerase-based linear amplification method were used to determine genomic locations [van Bakel et al. 2008]. After quantification and normalization, the binding profiles were corrected for nonspecific signals generated by a ChIP–chip experiment of an untagged yeast strain.

An overview plot of a part of chromosome III illustrates that NC2 binding overlaps with TBP on the promoters of pol II transcribed genes [Fig. 1A]. Although Mot1p ChIP signals were lower in general, we observe a strong overlap of the binding profiles of Mot1p with NC2 and TBP. In contrast, Taf1p binding overlaps with only a subset of TBP-binding sites. However, these sites also contain NC2 and Mot1p. TBP-binding peaks were observed on genes corresponding to tRNAs and snoRNAs [Fig. 1A, Supplemental Fig. 1]. In contrast, little binding of Mot1p, NC2, Taf1p, or Spt20p is found at these RNA pol III-transcribed genes [Supplemental Fig. 1]. For subsequent analysis, we excluded binding signals within 2 kb of pol III transcribed genes, as the strong TBP binding in these regions would interfere with the analysis of adjacent pol II transcribed genes. The resulting data set contains 90% of all pol II transcribed genes [5865 in total]. To compare the genomic binding profiles of the different factors to the transcriptional state of pol II genes, we selected gene clusters ranging from low to high mRNA expression levels [Holstege et al. 1998; Pokholok et al. 2005]. For each cluster we computed the average binding profile for the factor to the ORF (5′ end, middle, and 3′ end), promoter [fragments: −300/−551, −550/−301, or −300/−50 relative to the ATG start codon] and 3′ end region (200 bp downstream from the ORF). As expected, pol II localizes mainly to the ORF and 3′ end of genes and pol II binding strongly correlated with mRNA levels [Fig. 1B]. TBP binding peaks at the −300/−50 region encompassing the core promoter and at the 3′ end of the ORF. Similar to pol II, TBP binding correlates well with the transcription rate [Fig. 1C]. Taf1p [Fig. 1D] and Spt20p [Fig. 1E] display similar binding patterns as TBP, but we noted that Spt20p binding was shifted upstream. Similar to TBP, binding of Mot1p [Fig. 1F] and the NC2α and NC2β subunits [Fig. 1G,H] peaks at the core promoter region (~300/−50). Surprisingly, the binding of these negative regulators of TBP shows a strong positive correlation with mRNA expression levels. In conclusion, our genome localization data indicate that Mot1p and NC2 localize mostly to promoters of actively transcribed genes.

Genomic binding profiles of TBP, NC2α, NC2β, and Mot1p are overlapping

To investigate the relation between the TBP regulatory factors in more detail we performed an unsupervised hi-
ChIP-chip analysis of TBP complexes

Figure 1. ChIP-chip analysis of TBP, NC2α, NC2β, TBP, Mot1p, Taf1p, Spt20p, and pol II and the correlation to gene expression levels. [A] Overview plot of a part of chromosome III (115–165 kb) of the different binding profiles is shown. The locations of the oligos on the array are indicated in red and gene annotations in blue. The binding profiles are presented on the same scale (one- to fourfold over background). The location of the promoters for the pol II-transcribed PGK1, and for the pol III-transcribed n(NGUUC)III-transcribed genes are indicated at the top of the figure. Please note that as expected (Kuras et al. 2000) low levels of Taf1p can be detected at the TAF-independent PGK1 promoter. (B–H) Average binding analysis of pol II, TBP, Taf1p, Spt20p, Mot1p, NC2α, and NC2β for gene groups with <1, 1–4, 4–16, 16–50, or >50 mRNA copies per cell. Average binding profiles were determined for regions in the ORF (5′ end, middle, and 3′ end), promoter [fragments: −800/−551, −550/−301 or −300/−50 relative to the ATG start codon] and 3′ end region (200 bp downstream from the ORF).

erarchical clustering on the average promoter binding profiles of TBP, NC2α, NC2β, Mot1p, Spt20p, and Taf1p [Fig. 2A]. This reveals that the binding profiles of the NC2 subunits closely cluster together with Mot1p, but not with Taf1p or Spt20p. To examine the overlap in promoter binding of NC2, Mot1p, and TBP, two different binding cutoffs (more than twofold binding over input and a P-value <0.01 [Fig. 2B] and >1.5-fold binding over input and a P-value <0.05 [Fig. 2C]) were applied. When applying the stringent cutoff [Fig. 2B], NC2α localizes to almost 2800 promoters. In the less stringent statistical analysis, NC2α binds to ~3900 promoters [Fig. 2C]. By both criteria NC2β predominantly localizes to NC2α-bound promoters. Next, we compared Mot1p and TBP binding to promoters that carried either NC2α or NC2β. Almost all of Mot1p (>97%) and most of TBP-bound promoters (>83%) overlap with NC2-bound promoters. The outcome of this is comparable when NC2α and NC2β are analyzed separately [Supplemental Fig. 2A,B]. In addition, Taf1p and Spt20p binding profiles display a substantial overlap with NC2-, Mot1p-, and TBP-bound promoters [Supplemental Fig. 2C,D].

Next, we generated standard correlation plots for TBP,
NC2, and Mot1p to analyze resemblance of binding profiles. As expected, there is a substantial degree of correlation between NC2α and NC2β binding \( [R = 0.67] \), NC2α and Mot1p \( [R = 0.61] \), and NC2β and Mot1p \( [R = 0.64] \) [Supplemental Fig. 3A–C]. In contrast, the correlation between TBP and NC2α \( [R = 0.33] \), TBP and NC2β \( [R = 0.33] \), and TBP and Mot1p \( [R = 0.33] \) is more limited [Supplemental Fig. 3D–F]. This is expected, as TBP is also present in TFIIID and SAGA protein complexes and may bind to promoters independent of Taf1p. Thus, TBP binding represents the sum of the different protein complexes. To summarize, we find that NC2, TBP, and Mot1p colocalize on a large portion of promoters and that TFIIID or SAGA bind to a subset of the NC2- and Mot1p-bound promoters.

NC2β has a preference for TATA-less promoters

Previous studies have shown that basal transcription factors can have different preferences for promoters depending on the presence of a canonical TATA-box [Lemaire et al. 2000; Geisberg et al. 2002]. Global expression analysis indicated that TFIIID is important for the maintenance of transcription from promoters lacking a canonical TATA-box, whereas SAGA is important for TATA-box-containing promoters [Basehoar et al. 2004]. This prompted us to examine the TATA preference of the different factors included in this study. Among the TBP-, NC2α-, or NC2β-enriched promoters, ~20% contains a TATA-box [Fig. 2D], which is comparable with the genome-wide distribution of TATA-boxes [Basehoar et al. 2004]. In contrast, 27% of the Mot1p-bound promoters have a TATA-box, suggesting that Mot1p has a slight preference for TATA-box-containing promoters. Also, Spt20p preferentially binds to TATA promoters [31%], whereas Taf1p shows no enrichment of TATA promoters.

The analysis of Figure 2D only does not take into account whether promoter binding of a factor is strong or weak. To examine this in more detail, promoters were selected that have either strong or weak binding of Mot1p, NC2, TFIIID, or SAGA and calculated the binding ratio of each factor relative to TBP binding. As expected, promoters that have a relatively strong binding of Mot1p and Spt20p display an increased preference for TATA promoters [Fig. 2E]. Interestingly, in this analysis strong NC2α binding also correlates with a preference for TATA promoters. The fraction of TATA-containing promoters is reduced for genes that have a relatively high occupancy of NC2β and Taf1p. To examine the NC2β preference in more detail, relative occupancies of NC2α or Mot1p to NC2β were determined [Fig. 2F]. This reveals that TATA promoters are underrepresented when the binding of NC2α and Mot1p is low relative to NC2β. This indicates that NC2β, but not NC2α, has a binding preference for promoters lacking a canonical TATA-box.

Differential binding of the NC2 subunits during transcriptional reprogramming

The presence of NC2 and Mot1p on the promoters of transcribed genes suggests that they are directly involved in transcription. Given that TFIIID and SAGA are also present at these promoters, it is also possible that NC2 and Mot1p have a repressive function at active promoters. To examine interplay between activators and repressors more closely, we profiled the changes in binding patterns in response to changes in gene expression. To achieve this, mRNA expression profiles were determined in response to lowering glucose levels for 5 and 10 min [Fig. 3A, left panel]. This analysis indicates that 522 genes are up-regulated (>1.5-fold) [Fig. 3A, colored red] and 400 genes are down-regulated (>1.5-fold) [Fig. 3A, colored green] at 10 min after shifting from 4% to 0.1% glucose. ChIP samples from the different strains were prepared after the shift to low glucose in order to corre-

![Figure 3. Reprogramming of pol II, TBP, NC2α, NC2β, Mot1p, Taf1p, and Spt20p binding during a shift from 4% to 0.1% glucose-containing medium. (A) Cluster diagram of genes that significantly changed in expression at 10 min after shifting cells from 4% to 0.1% glucose. Red indicates activated genes and green indicates repressed genes. Based on the gene expression we selected six top-level clusters that were used for subsequent analysis in B. The corresponding change in binding was measured at 5 min after the low glucose shift for TBP, NC2α, NC2β, Mot1p, Taf1p, and Spt20p at promoters and pol II in the ORF. Red indicates increased binding and green decreased binding. (B) Average binding of TBP, NC2α, NC2β, Mot1p, Taf1p, Spt20p, and pol II at \( t = 0 \) [blue line] and \( t = 5 \) min [red line] within the six clusters that are indicated in A.](image-url)
late changes in transcription factor binding with expression changes. Our previous analyses (van Oevelen et al. 2005) indicate that the primary changes in transcription factor binding occur already 5 min after the glucose shift. Figure 3A (right panel) displays the average binding changes to the promoter region or to the ORF for pol II after the glucose shift. Clustering analysis of the expression data resulted in three clusters of gene activation (~2.3-, ~1.7-, and ~5.1-fold average increase in expression) and three clusters of gene repression (~1.5-, ~2.0-, and ~2.8-fold average decrease in expression). The transcription activation and repression in these clusters strongly correlate to average pol II binding [Fig. 3B]. For example, cluster 3 (activation ~5.1-fold) displays the largest increase of pol II binding (from onefold to 2.5-fold), whereas cluster 2 (activation ~1.7-fold) shows smallest increase in pol II binding. Changes in TBP binding also correlate well to transcription activation, with the largest increase of TBP binding in cluster 3. Strikingly, irrespective of the extent of repression, TBP is almost completely lost at all repressed genes. This suggests that there is a rapid reprogramming mechanism that actively removes TBP from promoters. Possibly, the different repression clusters originated from differences in pol II elongation rates and/or in mRNA half-lives. Interestingly, NC2α and NC2β are also recruited upon activation of transcription. In a similar analysis we find that Taf1p and Spt20p are recruited to activated promoters. This implies that there is a dynamic interplay between positive and negative regulators of TBP upon activation of transcription. At repressed genes, almost no loss of NC2α was detected, whereas NC2β binding is significantly reduced. Strikingly, Mot1p and Spt20p binding also do not change upon gene repression. This suggests that there are distinct roles of NC2α and NC2β during gene repression after shifting to low glucose.

Figure 4. TBP, NC2, and Mot1p copurify in chromatin extracts. (A) Chromatin extracts were isolated from C-terminal TAP-tagged Mot1p yeast cells and affinity-purified using a TAP-tag purification procedure. Eluates were separated on a SDS–polyacrylamide gradient gel, and Coomassie-stained bands were excised, subjected to in-gel tryptic digestion, and analyzed by mass spectrometry. The band labeled Mot1p was identified with 108 unique peptides with a 46% coverage. The labeled TBP, NC2α, and NC2β bands were identified with 17 (42% coverage), 19 (63% coverage), or 15 (54% coverage) unique peptides, respectively. The band labeled with one asterisk (*) is also present in the mock and represents Tef1p. The band labeled with two asterisks (**) mostly contained Mot1p and TBP derived peptides. In the band labeled with three asterisks (***) mostly NC2α peptides were found. (B) To determine the presence of DNA, part of the purification described in A was labeled in in a T4 polynucleotide kinase reaction using [γ-32P]ATP and loaded on a 20% polyacrylamide gel. The arrow indicates the position of the diffuse band. (C) Chromatin extracts were isolated from biotin-tagged TBP, NC2, or Mot1p strains expressing *Escherichia coli* BirA biotin ligase. Biotinylated proteins were immobilized using streptavidin beads. Input and eluates were analyzed by immublotting and probed for the indicated proteins. As a control, a nontagged strain expressing BirA was used. (D) To determine the presence of DNA, part of the samples described in C was eluted in TE buffer. Subsequently, samples were treated similarly to those described in B.
subunits, Taf4p and Taf1p, are also present in the TBP purification. Small amounts of pol II can be detected by CTD antibodies, suggesting that this is partly a transcriptionally active form of TBP. The results are comparable when we purified TBP via the HA tag [Supplemental Fig. 4B]. Mot1p, NC2, and TBP copurified with biotin-tagged NC2α or NC2β [Fig. 4C, lanes 8,9]. Interestingly, pol II and TFIIID subunits could not be detected. In line with these data, TBP, NC2α, and NC2β, but not pol II or TFIIID, copurifies with biotin-tagged Mot1p [Fig. 4C, lane 10]. Histone H3 was not detected in any of the samples [Fig. 4C, lanes 6–10]. Analysis of a nontagged control strain further confirmed that the detected signals are specific [Fig. 4C, lane 6]. DNA labeling of these samples results in a comparable DNA pattern as for affinity-purified Mot1p [Fig. 4D, lanes 3–6]. Dnase I treatment after the labeling reaction eliminates this pattern, verifying the presence of DNA [Supplemental Fig. 4C]. These data show that NC2, TBP, and Mot1p can form a complex on DNA in vivo.

ATP dependent dissociation of NC2 from TBP and Mot1p

An important function of Mot1p is its ability to remove TBP from DNA [Auble et al. 1994]. For this action Mot1p uses its intrinsic [d]ATPase activity. To test whether ATP hydrolysis disrupts the TBP, NC2, and Mot1p interactions, the TBP–Mot1p–NC2–DNA complex was purified from chromatin extracts and incubated with ATP or a nonhydrolyzable ATP analog [ATP-γ-S] [Fig. 5A]. Upon ATP treatment, Taf1p and Mot1p association with TBP remained unchanged [Fig. 5B, lanes 2–7], whereas binding of NC2α and NC2β is strongly decreased [Fig. 5B, lanes 3,5,7]. In agreement with this, binding of NC2α and NC2β, but not of TBP, is reduced after ATP addition in samples purified via tagged Mot1p [Fig. 5C, lane 4]. Conversely, an ATP dependent reduction in Mot1p and TBP is observed when the complex was purified via NC2α or NC2β. Interestingly, ATP addition results in partial disruption of the NC2 heterodimer [Fig. 5C, lanes 5–12]. Part of each sample was processed for DNA analysis. Addition of ATP to the Mot1p, NC2α-, and NC2β-purified complexes results in a loss of >60% of the DNA [Fig. 5D, lanes 1–9]. In contrast, the amount of DNA in the TBP sample is only reduced by 30% [Fig. 5D, lanes 10–12]. This is expected, as this DNA also represents TFIIID–DNA or TBP–DNA complexes, which would be resistant to ATP treatment [Fig. 5B]. These results suggest that Mot1p is involved in active disruption of NC2-TBP-DNA interactions, and perhaps can also dissociate the NC2α and NC2β heterodimer.

TBP and NC2 dissociation kinetics are delayed in mot1-1 mutant

In line with our in vitro data, it has been found that promoter binding of NC2 is increased in a mot1-1 mutant strain [Geisberg et al. 2002]. We decided to examine the role of NC2 and Mot1p in TBP dissociation during transcriptional shut-off in vivo. This was tested by analyzing their binding to the HXT2 promoter. HXT2 encodes a high-affinity hexose transporter, whose expression is highly responsive to changes in glucose concentration [Ozcan and Johnston 1995]. HXT2 expression was first induced by shifting the yeast cells from 4% to 0.1% glucose. Next, glucose was added back to 4% to repress the gene, and samples were taken after 1, 3, or 10 min. To reduce variation between samples, we carried out the ChIPs from a single chromatin extract using antibodies against TBP, NC2α, NC2β, and pol II. A primer set corresponding to the core promoter region of HXT2 (–170/–76) was used, and the signals were normalized to a fragment of the silent HMR locus. In wild-type cells TBP dissociation from the HXT2 promoter is complete in less than 1 min [Fig. 6A]. In contrast, TBP binding in the mot1-1 mutant is reduced to 50% only after 3 min. Similarly, dissociation of pol II is delayed [Fig. 6B]. Upon HXT2 transcription activation, binding of NC2α and
Wild type. In the mot1-1/H9251/H9251 for TBP, most of NC2 tagged NC2/H9252 mutant yeast strains were difficult to detect, we used biotin-display a delayed dissociation rate in the (Supplemental Fig. 5A,B). In conclusion, TBP and NC2 HMR identification of isolated DNA. A fragment of the responding to the core promoter of −mutant cells were increased in mot1-1 mutant, TBP and the NC2 subunits, and DNA, depending on ATP hydrolysis. This characterized a Mot1p–NC2–TBP–DNA complex from chromatin extracts, which dissociates into Mot1p–TBP, NC2 subunits, and DNA, depending on ATP hydrolysis. This is consistent with the observation that NC2 binding is increased in mot1 loss-of-function mutants (Geisberg et al. 2002). Together, our observations indicate that NC2 and Mot1p coordinately act to regulate the output of active promoters in yeast. These findings have important ramifications for the dynamic interplay between negative [NC2 and Mot1p] and positive regulators [TFIID and SAGA] of TBP function in transcription initiation.

Our findings are consistent with a model in which Mot1p and NC2 cooperate to restrict TBP activity stimulated by TFIID and SAGA, thereby the transcriptional output of active genes. On promoters of active genes ATP hydrolysis by Mot1p would act to dissociate the transcriptionally inert Mot1p–NC2–TBP complex from the promoter to allow association of TFIID or free TBP, which would direct productive pre-initiation complex assembly and subsequent transcription (Fig. 7). This dynamic exchange model is supported by the following observations. First, the genomic binding profiles of Mot1p, NC2, and TBP are largely overlapping (Fig. 2A,B). Second, NC2 and Mot1p binding positively correlates with promoter activity [Fig. 1F–H]. Third, SAGA and TFIID association to promoters overlaps with NC2 and Mot1p binding [Fig. 2A, Supplemental Fig. 2C,D]. Fourth, both NC2 and TFIID association is increased upon activation of promoters [Fig. 3A]. And, lastly, consistent with sequential ChIP experiments [Geisberg and Struhl 2004] no TFIID, pol II [Fig. 4C], or TFIIB [data not shown] could be detected in the Mot1p–NC2–TBP–DNA complexes isolated from chromatin, indicating that this represents a transcriptionally inactive complex.

An attractive feature of this dynamic exchange model is that on active promoters TBP can be kept in a equilibrium of active [TFIID or free TBP] and inactive [NC2–Mot1p] forms. Altering the activities of the TBP regulatory complexes would allow a very rapid adjustment of the transcriptional response. Indeed, cellular stress conditions have been shown to alter the properties of Mot1p–TBP complexes [Geisberg and Struhl 2004]. The dynamic exchange model is also consistent with observations that transcription in eukaryotic cells occurs in a discontinuous manner with intermittent pulses of transcription. The pulses are irregular in length and spacing and they are referred to as transcriptional bursts [Chubb et al. 2006; Raj et al. 2006]. During these bursts TFIID or free TBP would be occupying the promoter directing transcription, while between bursts the promoter would be occupied by the inactive Mot1p–NC2–TBP complex. Interestingly, the sequence of the TATA-box has been found to determine variability of transcriptional bursts [Blake et al. 2006].

Discussion

Here we present a detailed map of the genome wide localization of TBP, NC2, Mot1p, SAGA, TFIID, and pol II for yeast cells grown in exponential phase and during a shift from high to low glucose. We isolated and characterized a Mot1p–NC2–TBP–DNA complex from chromatin extracts, which dissociates into Mot1p–TBP, NC2 subunits, and DNA, depending on ATP hydrolysis. This is consistent with the observation that NC2 binding is increased in mot1 loss-of-function mutants (Geisberg et al. 2002). Together, our observations indicate that NC2 and Mot1p coordinately act to regulate the output of active promoters in yeast. These findings have important ramifications for the dynamic interplay between negative [NC2 and Mot1p] and positive regulators [TFIID and SAGA] of TBP function in transcription initiation.

Our findings are consistent with a model in which Mot1p and NC2 cooperate to restrict TBP activity stimulated by TFIID and SAGA, thereby limiting the transcriptional output of active genes. On promoters of active genes ATP hydrolysis by Mot1p would act to dissociate the transcriptionally inert Mot1p–NC2–TBP complex from the promoter to allow association of TFIID or free TBP, which would direct productive pre-initiation complex assembly and subsequent transcription (Fig. 7). This dynamic exchange model is supported by the following observations. First, the genomic binding profiles of Mot1p, NC2, and TBP are largely overlapping (Fig. 2A,B). Second, NC2 and Mot1p binding positively correlates with promoter activity [Fig. 1F–H]. Third, SAGA and TFIID association to promoters overlaps with NC2 and Mot1p binding [Fig. 2A, Supplemental Fig. 2C,D]. Fourth, both NC2 and TFIID association is increased upon activation of promoters [Fig. 3A]. And, lastly, consistent with sequential ChIP experiments [Geisberg and Struhl 2004] no TFIID, pol II [Fig. 4C], or TFIIB [data not shown] could be detected in the Mot1p–NC2–TBP–DNA complexes isolated from chromatin, indicating that this represents a transcriptionally inactive complex.

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NC2 and Mot1p reside on active genes

Our results extend earlier proposals of NC2 and Mot1p as transcriptional repressors [for review, see Pugh 2000]. A substantial amount of yeast genetic data have been accumulating, which supports a repressive function for the Mot1p and NC2 complexes (e.g., Davis et al. 1992; Prelich 1997; Lee et al. 1998; Peiro-Chova and Estruch 2007). The first indications for positive functions for Mot1p and NC2 came from in vitro transcription experiments showing that under certain conditions addition of these proteins stimulates transcription [Muldrow et al. 1999; Willy et al. 2000]. mRNA expression profiling studies of Mot1p and NC2 mutant strains also indicated positive functions for these complexes [Andrau et al. 2002; Cang and Prelich 2002; Dasgupta et al. 2002; Geisberg et al. 2002]. These were supported by ChIP studies on selected genes, which indicated that Mot1p and NC2 are recruited to promoters upon gene activation [Geisberg et al. 2001, 2002; Andrau et al. 2002; Dasgupta et al. 2002; Geisberg and Struhl 2004; Zanton and Pugh 2004]. Our study now expands this analysis to the entire genome, showing that binding of Mot1p and both NC2 subunits positively correlates with gene activity rather than with gene repression. Our localization results for yeast NC2 are supported by a recent study of human cells, which indicated that human NC2α is also localized to a large number of active promoters [Albert et al. 2007]. The overlapping profiles of NC2- and Mot1p with TBP support the view that NC2 and Mot1p require TBP for association to pol II promoters [Geisberg et al. 2002]. It is interesting to note that promoters with a high ratio of NC2 and Mot1p to TBP are also enriched for TFII D and SAGA [data not shown]. Surprisingly, these genes are expressed at a lower level, which suggests that this set of genes is highly regulated. In line with this, we found that many of these promoters have a high histone turnover rate [Dion et al. 2007; data not shown]. By analyzing a selected set of promoters it was found that the DNA surrounding the TATA-box plays an important role for Mot1p function, as the TATA-less promoters of HIS3 and HIS4 are more dependent on Mot1p than the canonical TATA promoters [Collart 1996; Geisberg et al. 2002]. In contrast, microarray mRNA expression analysis indicated that Mot1p functions to repress TATA promoters [Basehoar et al. 2004]. In the Mot1p binding profile we find a modest enrichment for TATA promoters [Fig. 2D]. This does not correspond to the relaxed specificity of Mot1p–TBP and human BTF1–TBP complex [Gumbs et al. 2003; Klejman et al. 2005]. Similar to Mot1p, SAGA-enriched promoters display a slightly higher proportion of TATA-boxes [Fig. 2D], which is consistent with the observation that TATA-box promoters preferentially use SAGA [Basehoar et al. 2004]. Together, this supports previous models that Mot1p and SAGA collaborate in regulating gene expression [Collart 1996; Madison and Winston 1997; van Oevelen et al. 2005].

Differential roles of the NC2 subunits

The genomic localization profiles of NC2α and NC2β are largely overlapping during normal growth, which indicates that these proteins work as a complex. Previous ChIP analyses indicated that, upon diauxic shift, the two subunits may play different functions. A relative enrichment of NC2α was observed on activated promoters, and NC2β was more abundant on repressed promoters [Creton et al. 2002]. In contrast, we find that, upon glucose-induced transcriptional reprogramming, both NC2α and NC2β are recruited to activated promoters. Fifty percent of these activated promoters bear a canonical TATA-box. The extent of NC2β recruitment to the activated promoters seems less compared with NC2α [Fig. 3B], and NC2β is slightly underrepresented on TATA promoters [Fig. 2E,F]. Previous analyses showed that transcription from the TATA-less promoter of HIS3 requires functional NC2α and NC2β [Lemaire et al. 2000]. Clearly, more experiments are needed to elucidate the selective functions of NC2α and NC2β.

It is interesting to note that ChIP–reChIP experiments showed that Mot1p and TFII B can co-occupy activated promoters during heat shock [Geisberg and Struhl 2004]. Structural analysis of the human TBP–NC2 complex indicates that NC2β and TFII B binding are mutually exclusive [Kamada et al. 2001]. And a mutational study of human TBP indicated that the BTAF1 and TFII B interact with different residues of human TBP [Klejman et al. 2005]. Possibly, selective NC2β dissociation from the NC2–Mot1p–TBP promoter complex induced by heat shock allows TFII B entry to direct productive transcription. It is possible that the relative differences in NC2 subunit association during promoter activation, e.g., induced by low glucose shift or heat shock, represent transient effects. As they are most prevalent during transcriptional reprogramming, this suggests regulation of Mot1p-mediated dissociation of NC2–Mot1p–TBP–promoter complexes.

Concluding remarks

Our genomic localization study now provides an explanation for the seemingly contradictory results for NC2 and Mot1p obtained from biochemical and mRNA profiling experiments. Whereas previous models indicated that Mot1p and NC2 compete for binding to TBP-DNA complexes [Geisberg et al. 2002], we now propose that Mot1p and NC2 cooperate in a dynamic manner to restrict TBP activity stimulated by SAGA and TFII D to limit transcription levels. This dynamic exchange model provides a framework for further experiments regarding the interplay of TBP regulatory proteins.

Materials and methods

Strains and plasmids

NC2α and NC2β were tagged with the biotin-acceptor-sequence (avitag) at the N terminus and Mot1p, Spt20p, and Taf1p at the C terminus in wild-type W303B (MATa ade2-1 tral-1 his3-11,15 leu2-3,112 trp1-1 can1-100) as described previously [van Werven and Timmers 2006]. Strains used in this study are described in Supplemental Table 1. In order to achieve biotinylation of the avitag, strains were transformed with pRS313-
ChIP

ChIP has been carried out as described previously (van Werven and Timmers 2006). In short, 400 µL of mid-log growing yeast cells (OD$_{600}$ = 0.4) were cross-linked with 1% formaldehyde for 20 min at room temperature. The reaction was quenched with glycine, and cells were collected by centrifugation. Subsequently, cells were disrupted using a beadbeater and sonicated (Bioruptor, Diagenode: seven cycles, 30 sec on/off, medium setting) to produce an average DNA fragment size of 400 bp. For the immunoinulation tag ChIPs, 500 µL of extract were incubated with 80 µL of Dynabeads M-280 Streptavidin [Invitrogen] for 1 h and 45 min. Samples were subsequently washed three times with 0.5 M LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% Na-deoxycholate and three times with 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 3% SDS. For the antibody ChIPs in Figure 6, 2.5 µL of antibodies (CTD, TBP, NC2a, and NC2b) were coupled to 30 µL Protein A Dynabeads [Invitrogen]. After incubation with 500 µL of extract the antibody ChIPs were washed three times in FA lysis buffer (50 mM HEPES KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS) and three times with FA lysis buffer containing 0.5 M NaCl. Cross-links of the ChIP samples was reversed overnight at 65°C in 150 µL 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% SDS. Samples were treated with proteinase K, and DNA was recovered for further analysis.

Microarray expression profiling

W303B wild-type strain was grown in synthetic complete (SC) medium containing 4% glucose till OD$_{600}$ = 0.4. For the low glucose shift, cells were collected by centrifugation and suspended in SC containing 0.1% glucose. Samples were taken after 5 and 10 min. Total RNA was isolated as described (van Oevelen et al. 2005). Samples were subsequently amplified using the T7 amplification method, labeled with Cy3 or Cy5 dyes, and hybridized to yeast oligo microarray arrays. Each time point was grown, amplified, and hybridized in quadruplicate against a common reference pool. Slides were scanned and normalized, and P-values were computed as described (van de Peppel et al. 2008). Probes with <$0.05$ value and average 1.5-fold change were considered as significantly changed. For the clustering analysis Genespring 7.0 [Agilent] was used.

ChIP–chip amplification, labeling, and hybridization

Samples were amplified using a double round T7 based amplification procedure as described previously (van Bakel et al. 2008). In short, in a terminal transferase reaction a poly-dT was generated at the 3' end of the DNA. In a klenow fill-in reaction the poly-dT was used as a template for the T7-[dA]$_{18}$ oligo. Next, samples were amplified using MEGAscript T7 kit [Ambion]. After the first round, samples were reverse transcribed using random primers followed by a klenow fill in reaction using T7-[dA]$_{18}$ oligo. Similar to the first round, samples were amplified using MEGAscript T7 kit [Ambion] except that 3-aminoallyl-UTP was used in the reaction. Amplified samples were labeled using nonfunctional NHS-ester Cy3 or Cy5 dye [GE Healthcare] and hybridized to oligonucleotide arrays [Agilent Technologies] that contain 60-mer oligonucleotide probes covering the complete yeast genome at an average of 266-bp resolution. The slides were washed and scanned accordingly. Genome-wide localization analysis data were generated from two biological samples that were differentially labeled [Cy3 or Cy5] and hybridized independently.

ChIP–chip data analysis

Following quantification, the microarray data was normalized using a density lowess-normalization algorithm [for detailed description see the Supplemental Material]. The binding enrichment was computed by dividing the normalized chip signal over the input signal. With use of MAANOVA statistical package, P-values were determined by a permutation F2 test in which residuals were shuffled 1000 times. A combination of P-values and binding ratio cutoffs was used to identify significantly bound genomic regions. Average binding analysis was carried out as described by van Bakel et al. (2008).

Preparation of chromatin extracts

Chromatin extracts were prepared essentially as described [Vermeulen et al. 2007]. Briefly, cells were disrupted using glass beads in nucleosome isolation buffer [NIB] 0.1% Triton X-100, 10 mM MgCl$_2$, 20 mM HEPES NaOH [pH 7.8], 250 mM sucrose. The pellet was collected by centrifugation at 15,000 rpm for 15 min at 4°C. This pellet was washed and resuspended in NIB plus 2 mM CaCl$_2$. Next, samples were treated with 8 units of micrococcal nuclease per 1 mL extract [Sigma] for 2 min at 30°C. The reaction was stopped with 10 mM EGTA [pH 8.0]. The NaCl concentration of each sample was adjusted to 150 mM before samples were centrifugated at 14,000 rpm for 5 min at 4°C. The supernatant represents the chromatin extract. In these extracts nucleosomal particles are the most abundant protein–DNA complex, as evidenced by the prominence of nucleosomalized DNA of ~150 bp.

Immunoprecipitation

One liter of cultured yeast cells (OD$_{600}$ = 0.7) was collected by centrifugation from which a chromatin extract was prepared. For the immunoprecipitation 1 mL of chromatin extracts were incubated with 20 µL of Dynabeads M-280 Streptavidin [Invitrogen] or 20 µg anti-HA [12CA5] antibodies coupled to 20 µL Protein A Dynabeads [Invitrogen] for 2 h at 4°C. Samples were then washed six times with NIB buffer plus 150 mM NaCl and eluted by incubating for 5 min at 95°C in sample buffer for immunoblot analysis or 10 mM Tris-HCl [pH 8.0], 1 mM EDTA for labeling of DNA.

Radioactive DNA labeling

DNA isolated during the immunoprecipitation experiments was treated with 1 µL of shrimp alkaline phosphatase [SAP] [Roche] for 1 h at 37°C followed by inactivation of the enzyme at 65°C for 20 min. Samples were radioactively labeled in a reaction containing kinase buffer, 5 U of T4 polynucleotide kinase [New England Biolabs], and 25 µCi of [$^{32}$P]ATP for 30 min at 37°C. The labeled material was treated with 0.5 µL RNase [10 mg/mL] and sonicated till 70% of polycrylamide gel. The gel was dried and exposed to a PhosphorImager screen for analysis and quantification with a Storm 820 scanner [Molecular Dynamics] using ImageQuant TL software.

TAP purification

One liter of wild-type or Mot1-TAP-tagged cells were grown in YPD till OD$_{600}$ = 3 and were collected by centrifugation. Approximately 12 mL of chromatin extract was prepared from
these cell pellets. The chromatin extracts were incubated with 200μL IgG sepharose fast flow column [Pharmacia] for 2 h at 4°C on a rotating platform. The samples were subsequently treated according to a standard TAP tag procedure as described [Rigaut et al. 1999]. Purified proteins were concentrated as described [Wessell and Flugge 1984].

Protein identification by mass spectrometry

Mass spectrometry analysis was performed as described previously [Mousson et al. 2007]. Selected bands from the SDS/PAGE gel were digested using sequencing grade trypsin [Roche] and analyzed using LTQ-FTICR [Thermo Fisher Scientific] mass spectrometry. LTQ-FTICR data were searched, using an in-house-licensed mascot [Matrix Science] search engine, against the Yeast SGD database with carbamidomethyl cysteine as a fixed modification and oxidized methionines as variable modification. The mass tolerance of the precursor ion was set to 3 ppm and that of fragment ions to 0.6 Da. Scaffold [Proteome Software] was used to validate protein identifications. Protein identifications were accepted if they could be established at >99.9% probability and contained at least two identified peptides. For detailed description of the method see Supplemental Material.

Accession numbers

The raw and normalized ChIP-chip and microarray expression data have been submitted to the public microarray ArrayExpress database with accession numbers E-MTAB-21 and E-MTAB-22, respectively. Mass spectrometry protein data have been submitted to the proteomics identifications database [PRIDE] with accession numbers 3355–3368.

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Cooperative action of NC2 and Mot1p to regulate TATA-binding protein function across the genome

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