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The dual control of TFIIB recruitment by NC2 is gene specific

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ABSTRACT

Negative co-factor 2 (NC2) is a conserved eukaryotic complex composed of two subunits, NC2α (Drap1) and NC2β (Dr1) that associate through a histone-fold motif. In this work, we generated mutants of NC2, characterized target genes for these mutants and studied the assembly of NC2 and general transcription factors on target promoters. We determined that the two NC2 subunits mostly function together to be recruited to DNA and regulate gene expression. We found that NC2 strongly controls promoter association of TFIIB, both negatively and positively. We could attribute the gene-specific repressor effect of NC2 on TFIIB to the C-terminal domain of NC2β, and define that it requires ORF sequences of the target gene. In contrast, the positive function of NC2 on TFIIB targets is more general and requires adequate levels of the NC2 histone-fold heterodimer on promoters. Finally, we determined that NC2 becomes limiting for TATA-binding protein (TBP) association with a heat inducible promoter under heat stress. This study demonstrates an important positive role of NC2 for formation of the pre-initiation complex on promoters, under normal conditions through control of TFIIB, or upon activation by stress via control of TBP.

INTRODUCTION

Transcription by RNA polymerase II is critically dependent upon general transcription factors (GTFs) that allow the specific association of the polymerase with promoter regions. Amongst these, the TATA-binding protein (TBP) binds to promoters and plays a critical role in the nucleation of the pre-initiation complex (PIC) (1). It allows the recruitment of both TFIIA and TFIIB, followed by the other GTFs. Several factors that control transcription initiation, interact with TBP and either modify the association of TBP with DNA, or prevent the association of subsequent GTFs. One such factor is negative cofactor 2 (NC2), bearing two subunits (NC2α or Drap1 and NC2β or Dr1), which forms a stable complex with TBP on promoters (2). Biochemical and genetic data have suggested that the association of NC2 with DNA-bound TBP competes with the association of TFIIA and TFIIB, and thus inhibits transcription initiation (3–8). NC2 is conserved in eukaryotes, and a crystal structure of a human NC2–TBP–DNA complex has been resolved (8). NC2 dimerizes through histone-fold domains (HFD) of the H2A/H2B type, and the NC2 histone-fold is localized underneath the DNA surface to which TBP binds. Originally it was suggested that the carboxy terminal extension of NC2β might sterically hinder the association of TFIIB with TBP, whilst regions of NC2α missing in the structure, C-terminal to the HFD, might be responsible for sterically affecting the association of TFIIA with TBP. In contrast to the mutually exclusive binding to TBP proposed for both TFIIA and NC2, a more recent superposition of structures has suggested that TFIIA and NC2 could be bound to TBP simultaneously, albeit with lower affinity than for either molecule alone (9).

In addition to this simple and quite well-defined model for transcriptional repression by NC2, many studies have revealed that NC2 function is complex. Indeed, NC2 has been demonstrated not only to repress, but also to activate transcription, *in vitro* and *in vivo* (6,10–13). The mechanism by which NC2 promotes transcription has not been studied much, and remains unclear. The C-terminal domain of *Drosophila* NC2β, essential for repression by NC2, is not required for activation by NC2 (11). This observation would suggest the existence of different functional domains within NC2. A different study has suggested that different functional forms of NC2 might exist. Indeed, purification of NC2α and NC2β subunits from *Saccharomyces cerevisiae* revealed that the two NC2 subunits could not be co-purified from yeast cells growing exponentially, whilst they could be co-purified after glucose depletion (14). Furthermore, relative cross-linking of the NC2 subunits to a same promoter was different before and

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Table 1. Strain list

Strain name	Genotype	Reference
MY1	*MATa gcn4Δ ura3-52 trp1Δ1 leu2::PET56 gal2*	(14)
MY3298	Isogenic to MY1 except *MATα his3::TRP1 bur6-5*	This study
MY3357	Isogenic to MY1 except *MATα ncb2-2*	This study
MY3717	Isogenic to MY1 except *bur6-5 tfa1::TFA1-MYC$_{10}$-KanMX4*	This study
MY3718	Isogenic to MY1 except *tfa1::TFA1-MYC$_{10}$-KanMX4*	This study
MY3767	Isogenic to MY1 except *MATα ncb2-2 tfa1::TFA1-MYC$_{10}$-KanMX4*	This study
MY3765	Isogenic to MY1 except *bur6-5 tfb3::TFB3-MYC$_{10}$-KanMX4*	This study
MY3721	Isogenic to MY1 except *tfb3::TFB3-MYC$_{10}$-KanMX4*	This study
MY3802	Isogenic to MY1 except *MATα ncb2-2 tfb3::TFB3-MYC$_{10}$-KanMX4*	This study
MY4401	Isogenic to MY3298 except *sua7::SUA7-MYC$_{10}$-KanMX4*	This study
MY4312	Isogenic to MY1 except *sua7::SUA7-MYC$_{10}$-KanMX4*	This study
MY5130	Isogenic to MY1 except *MATα ncb2-2 sua7::SUA7-MYC$_{10}$-KanMX4*	This study
MY5661	Isogenic to MY1 except *hsp12::TRP1 sua7::SUA7-MYC$_{10}$-KanMX4*	This study
MY5662	Isogenic to MY5661 except *ncb2-2*	This study
MY5709	Isogenic to MY1 except *rps14b::TRP1 sua7::SUA7-MYC$_{10}$-KanMX4*	This study
MY5710	Isogenic to MY5709 except *ncb2-2*	This study

after glucose depletion suggesting that different forms of NC2 complexes might be able to associate with promoter DNA. What these different forms are remains unknown. In human, evidence has been provided to suggest that NC2α and NC2β can associate with different proteins, since it was demonstrated that BTAF1, the human homolog of yeast Mot1p, interacts with NC2α but not NC2β or the NC2 heterodimer (15). With regard to the mechanism by which NC2 might associate with DNA, it has been documented that NC2 associates with DNA-bound TBP, and this has been studied *in vitro* using TATA-containing DNA, but efficient binding of NC2–TBP to DNA that lacks a canonical TATA box has also recently been demonstrated (16). This observation may be related to former experiments showing that in yeast, NC2 was required for transcription from the TATA-less promoter of *HIS3* but repressed transcription from the *HIS3* TATA promoter (13). Finally, while the activity of NC2 has generally been thought to be related to TBP function, it was recently demonstrated *in vitro* that recombinant human NC2, like other histone-fold complexes, could facilitate nucleosome assembly by ACF, independently of a direct interaction with ACF (17). Furthermore, *Drosophila* NC2β has been described as a partner of the HFD protein TAF11 in two hybrid experiments (18).

In this work, using mutants of NC2, we have been able to define NC2 target genes, and determine how NC2 controls the assembly of the PIC on target genes *in vivo*. We demonstrate that in cells growing exponentially, NC2 regulates the association of TFIIB to promoters in different ways depending upon the gene. Furthermore, our study defines a role for NC2 in the stable recruitment of TBP to heat inducible genes upon heat stress. Taken together, our results reveal an important role for the NC2 heterodimer in the efficient assembly of GTFs to promoters.

MATERIALS AND METHODS

Strains and growth media

The strains used in this study are listed in Table 1. All media were standard, and YPD was used for glucose-rich medium. *HSP12* and *RPS14b* were disrupted by transformation of a PCR-amplified marker *TRP1* cassette according to Longtine (19).

Microarrays

Wild-type (wt) and mutant cells were grown exponentially at 30°C in YPD (2% glucose) during 24 h until an OD_{600} of ~0.8. Cells were harvested and total RNA extracted by the acid phenol method. The quality of total cellular RNA was tested on a RNA 6000 Nano Chip (Agilent). Total cellular RNA was next purified with RNeasy Mini Handbook kit (QIAGEN). Then, the synthesis of cDNA, cRNA, the hybridization and the scan of the chip were performed according to the technical manual of Affimetrix (GeneChip Expression Analysis). Briefly, oligo dT was added to 15 µg of purified total RNA. After 10 min. of incubation at 70°C in first strand buffer with DTT (10 mM), dNTP (500 µM each) and reverse transcriptase (SuperScript II from Invitrogen at 200 U/µl) were added for the first strand cDNA synthesis (1 h at 42°C). For the second strand synthesis, dNTP (200 µM each), *Escherichia coli* DNA ligase (10 U), *E. coli* DNA polymerase I (40 U) and *E. coli* RNaseH (2 U) were added to the second strand buffer and incubated for 2 h at 16°C. Finally, T4 DNA polymerase (10 U) was added to the reaction. The cDNA was purified and after ethanol precipitation, 15 µg of purified cDNA were incubated during 5 h at 37°C with HY buffer (Enzo), biotine-coupled ribonucleotides, DTT, an RNase inhibitor mix and T7 RNA polymerase. The cRNA was then purified in the same way as total RNA and 20 µg of cRNA were fragmented in 5× hybridization buffer (200 mM Tris–acetate pH 8.1, 500 mM KOAc, 150 mM MgOAc) and incubated during 35 min at 95°C.

Hybridization of cRNA. Fifteen micrograms of cRNA were hybridized to the Yeast Genome S98 chip, during 16 h at 45°C with 1× hybridization buffer (100 mM MES; 1 M [Na^+]; 20 mM EDTA; 0.01 Tween-20), oligonucleotide B2 (50 pM), eukaryotic hybridization controls (20×), herring sperm DNA (0.1 mg/ml) and acetylated BSA (0.5 mg/ml). The chip was washed in the Fluidics station, first with buffer A (6× SSPE, 0.01% Tween-20) then with buffer B (100 mM MES, 0.1 M [Na^+], 0.01% Tween-20).

Antibodies were added next (Normal Goat IgG 0.1 mg/ml; biotinylated antibodies 0.5 mg/ml; acetylated BSA 2 mg/ml; 100 mM MES; 1 M [Na^+]; 0.05% Tween-20) followed by the SAPE solution (Streptavidine–Phycoerythrine 10 μg/ml; acetylated BSA 2 mg/ml; 100 mM MES; 1 M [Na^+]; 0.05% Tween-20). Finally, the chip was scanned and the scan was analyzed by Affymetrix software (MicroArraySuite, MicroDB, and DataMiningTool) and Iobion software (Array Assist). The stringent analysis consisted of using (p0.05 DE1.0) to obtain significant target genes for each mutant compared to the wt by all three proposed methods of analysis (RMA, GCRMA and CHP). Only genes defined as genes significantly de-regulated in the mutant relative to the wt by all three analyses were considered. For comparison, a less-stringent analysis consisted of using the Affymetrix software, and looking for genes de-regulated in 100% of the comparisons (each mutant duplicate compared to each wt duplicate), without introducing an additional minimal fold increase or decrease. Once target genes for each mutant were defined by the Array Assist software, for each target gene we determined whether in each duplicate for each mutant it was expressed at higher levels than for either duplicate of the wt (Supplementary Table 1).

Chromatine immunoprecipitation (ChIP)

ChIP experiments were performed as described in (14). Briefly, wt and mutant cells were grown exponentially at 30°C in YPD (2% glucose) during 24 h until an OD_{600} of ~0.8. The cells were fixed with 1% formaldehyde during 20 min. at RT, and glycine was added to a final concentration of 125 mM to stop the reaction. The cells were washed twice (20 mM Tris–HCl pH 7.5; 200 mM NaCl) and broken in lysis buffer (50 mM HEPES–KOH pH 7.5; 140 mM NaCl; 1 mM EDTA pH 8; 1% Triton; 0.1% sodium deoxycholate; 1 mM PMSF) during 30 min and sonicated. The size of fragmented chromatin was verified on a gel to be between 200 and 400 bp, then a fraction of the extracts (input) was incubated over night at 4°C with specific antibodies and protein G-Sepharose. The Sepharose beads were collected and washed with TSE 150 (20 mM Tris–HCl pH 8; 2 mM EDTA pH 8; 1% Triton; 0.1% sodium deoxycholate; 150 mM NaCl), followed by TSE 500 (20 mM Tris–HCl pH 8; 2 mM EDTA pH 8; 1% Triton; 0.1% sodium deoxycholate; 300 mM NaCl), then Buffer III (10 mM Tris–HCl pH 8; 1 mM EDTA pH 8; 250 mM LiCl; 1% Igepal, 0.1% sodium deoxycholate) and finally 2 × TE. Immunoprecipitated chromatin was eluted in 1% SDS; 50 mM Tris–HCl pH 7.5; 10 mM EDTA pH 8. Then 10 mg/ml of proteinase K was added to the precipitates and to a fraction of the input during 5 h at 65°C to reverse cross-links. DNA was extracted and precipitated. Specific promoter DNA in the input and precipitates was measured by real-time PCR using SYBR Green (Eurogentec). The sequences of specific oligonucleotides are available upon request. 9E10 monoclonal anti-MYC antibodies were purchased from Covance, and mouse monoclonal anti-Rpb3p and anti-Rpb4p antibodies from Neoclone. The ChIP experiments were performed three times and the results are the fold (increase or decrease) change of the mutant versus the average wt value.

Co-immunoprecipitation experiments

Cultures were grown exponentially to an OD_{600} of 0.8. The equivalent of 50 OD_{600} of cells were harvested by centrifugation for 1 min at 13 000 r.p.m. After washing in water, cell pellets were frozen at −80°C, thawed and resuspended in 600 μl of buffer B (40 mM HEPES–KOH pH 7.5, 150 mM KOAc, 100 mM KCl, 20% glycerol, 1× protease inhibitor cocktail and 1 mM PMSF). Cells were broken by the addition of 300 μl of glass beads and vortexing vigorously for 15 min. Whole-cell extracts (WCE) were clarified by centrifugation for 15 min. at 13 000 r.p.m. and at 4°C. The protein concentration was determined by the Bradford assay. For co-immunoprecipitation experiments, 1 μl of anti-NC2α antibody was mixed with 1 mg of cell extract and 20 μl of protein G-Sepharose beads over night at 4°C in a total volume of 250 μl. The beads were washed three times with 40 mM HEPES–KOH pH 7.5, 150 mM KOAc, 100 mM KCl, 20% glycerol before elution with boiling in loading buffer.

S1 analysis

Total cellular RNA extraction and S1 analyses were performed as described previously (14). The sequences of the oligonucleotides used are available upon request.

RESULTS

C-terminal extensions following the HFDs of NC2α and NC2β are important for cell growth

Previous studies have suggested that the NC2 α- and β-subunits might have different roles in cells growing exponentially, and that the function of NC2 is regulated by glucose (14). Indeed, in yeast, differences in association of NC2 subunits with DNA, and changes in co-purification of the NC2 subunits, were observed between growth in high or low glucose. To further characterize the transcriptional functions of the two subunits of NC2, we generated two mutant strains. The first strain expresses from its endogenous locus and promoter, a NC2β mutant truncated at the C-terminus after the HFD and fused to a triple HA epitope (MY3357, bearing the *ncb2-2* allele and expressing the $NC2\beta_{\Delta 122}$ protein, Table 1). The second strain expresses from its endogenous locus and promoter a mutant NC2α truncated at the C-terminus after the HFD and fused to a triple HA epitope (MY3298, bearing the *bur6-5* allele and expressing the $NC2\alpha_{\Delta 120}$ protein, Table 1). Both mutant strains were cold and temperature sensitive for growth, and the *ncb2-2* mutant additionally grew slowly even at 30°C in rich medium (Figure 1A). We next analyzed the profile of transcripts genome-wide in wt, *bur6-5* and *ncb2-2* cells that we kept growing exponentially for 24 h in rich medium and high glucose (2%). We performed a stringent analysis that considered only genes whose

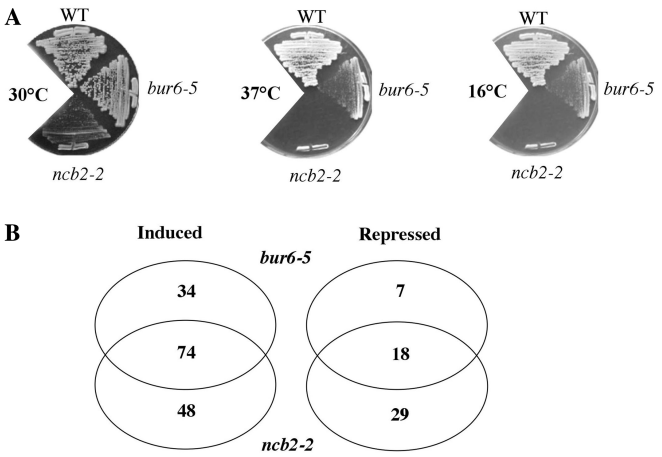

Figure 1. Analysis of *bur6-5* and *ncb2-2* growth and definition of target genes by microarray experiments. (**A**) The indicated strains were streaked on glucose-rich medium and placed at the indicated temperatures for 3–7 days. (**B**) Venn diagrams indicating the number of genes that were defined as induced or repressed in *bur6-5* or *ncb2-2* using the Array Assist software. The overlap between the two mutants was defined by determining for each of the target genes of one mutant whether each duplicate of the other mutant was different from each duplicate of the wild-type, with an average of 2-fold difference (data provided in Supplementary Table 1).

expression was significantly different in the mutant compared to the wt (Array Assist software, Iobion, see Materials and Methods section). This stringent analysis defined 133 target genes for the C-terminal deletion mutant of NC2α (*bur6-5*) and 169 target genes for the C-terminal deletion mutant of NC2β (*ncb2-2*) (Figure 1B and Supplementary Table 1). Genes were both up- and downregulated in the *bur6-5* and *ncb2-2* mutants, and there was a significant overlap in the genes de-regulated in both mutants (Figure 1B). These results support a strong interplay between both subunits since, in addition to the important overlap of target genes between the two mutants, many genes that were affected in the *ncb2-2* mutant only, are actually genes that respond to a change in growth rate such as *ADE12*, *ADE13* and *ADE17*, and only the *ncb2-2* mutant grew slowly under the conditions of analysis (see Figure 1A growth of *ncb2-2* versus wt and *bur6-5*).

Independent mRNA analysis confirms majority target genes identified by microarrays

To obtain confirmation of the results found by the microarray analyses, we analyzed by S1 digestion the mRNA levels of some of the target genes defined. In support of the microarray experiments, *BUR6*, the gene coding for NC2α itself, was upregulated in both mutants compared to the wt (Figure 2A). For the general stress genes *HSP12* and *HSP26*, the microarray data showed an induction in both mutants, whereas S1 analysis suggested a strong increase of *HSP26* mRNA in both mutants but only in the *ncb2-2* mutant for *HSP12* (Figure 2B and C). *MUC1* (also termed *FLO11*), encoding a cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth, is the gene whose mRNA was the most induced in the *ncb2-2* mutant according to the microarray experiments (around 77-fold increase). We could confirm a strong upregulation or this mRNA by S1 analysis (Figure 2D), and similarly we were able to confirm the upregulation of *DAN1* in the *ncb2-2* mutant (Figure 2E). We were also able to confirm decreased expression of several genes in the mutants compared to the wt: *RPS14b* (Figure 2F), *RPS28b*, *RPS9a* and *BNA4* (data not shown) as well as genes whose expression was not affected in any of the mutants such as *ADH1* or *PHO4* (Figure 2G and H).

Global loss of NC2 on promoters in the two mutants

The next step in our analysis of the mutants was to define what was happening with the NC2 subunits on the promoters of the target genes defined earlier. We thus compared the association of NC2α and NC2β to DNA in wt and mutant cells by ChIP experiments. We found that there was a significant reduction in cross-linking of both NC2 subunits to DNA, whether we checked genes upregulated in the mutants such as *HSP12* and *BUR6* (Figure 3A), genes downregulated in the mutants such as *RPS14b* (Figure 3A) or genes unaffected in the mutants (data not shown).

Since differences in cross-linking of both NC2 subunits to DNA in both mutant strains were observed, we next analyzed the expression and interaction of the NC2 subunits in wt cells and in cells expressing the mutant forms of the NC2 subunits (Figure 3B). In *bur6-5*, wt levels of NC2α could be immunoprecipitated with NC2α antibodies (lane 5, upper panel), despite lower levels of the protein in the total extract of mutant cells compared to wt cells (compare lanes 1 and 2, upper panel), but no detectable NC2β was co-immunoprecipitated (lane 5, lower panel), although NC2β was expressed at wt levels in this strain (compare lanes 1 and 2, lower panel). Furthermore, high levels of NC2β could be immunoprecipitated with NC2β antibodies in this strain (lane 8, lower panel), but only very low levels of NC2α co-immunoprecipitated (lane 8, upper panel). In *ncb2-2*, NC2β is expressed at only very low levels (lane 3, lower panel), and thus whether NC2α or NC2β is immunoprecipitated (lane 6, upper panel; or lane 9, lower panel), only very low levels of the other subunit is detectable in the immunoprecipitate (lane 9, upper panel and lane 6, lower panel). These results demonstrate that in both mutant strains, there are only low levels of the NC2 heterodimer, which correlates nicely with reduced cross-linking of both subunits to DNA in both strains, in contrast to wt expression of NC2β in *bur6-5* and NC2α in *ncb2-2*.

The general changes in transcription for the mutants

In order to analyze the status of the PIC on target promoters in the strains expressing the mutant forms of NC2, we investigated the presence of different factors of the transcription machinery using ChIP experiments. The first protein of the PIC we chose to look at was the TBP. Indeed, NC2 has previously been found to accelerate TBP binding to promoters and stabilize TBP–DNA complexes (16). Interestingly, we found that on *HSP12* no significant

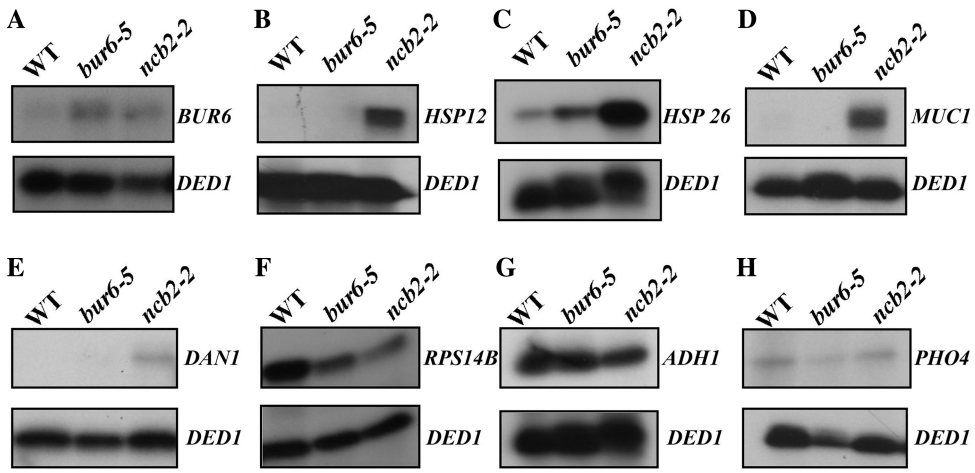

Figure 2. mRNA analysis to confirm the target genes found by the microarrays. Cells were grown up to an O.D.$_{600}$ of 0.8, and 50 µg of total cellular RNA was analyzed by S1 digestion for the levels of the indicated transcripts: (**A**) *BUR6*, (**B**) *HSP12*, (**C**) *HSP26* (**D**) *MUC1*, (**E**) *DAN1*, (**F**) *RPS14b* (**G**) *ADH1* and (**H**) *PHO4*. *DED1* were measured in each reaction as an internal control, since the microarray experiments indicated that it was not affected by the *bur6-5* or *ncb2-2* mutations.

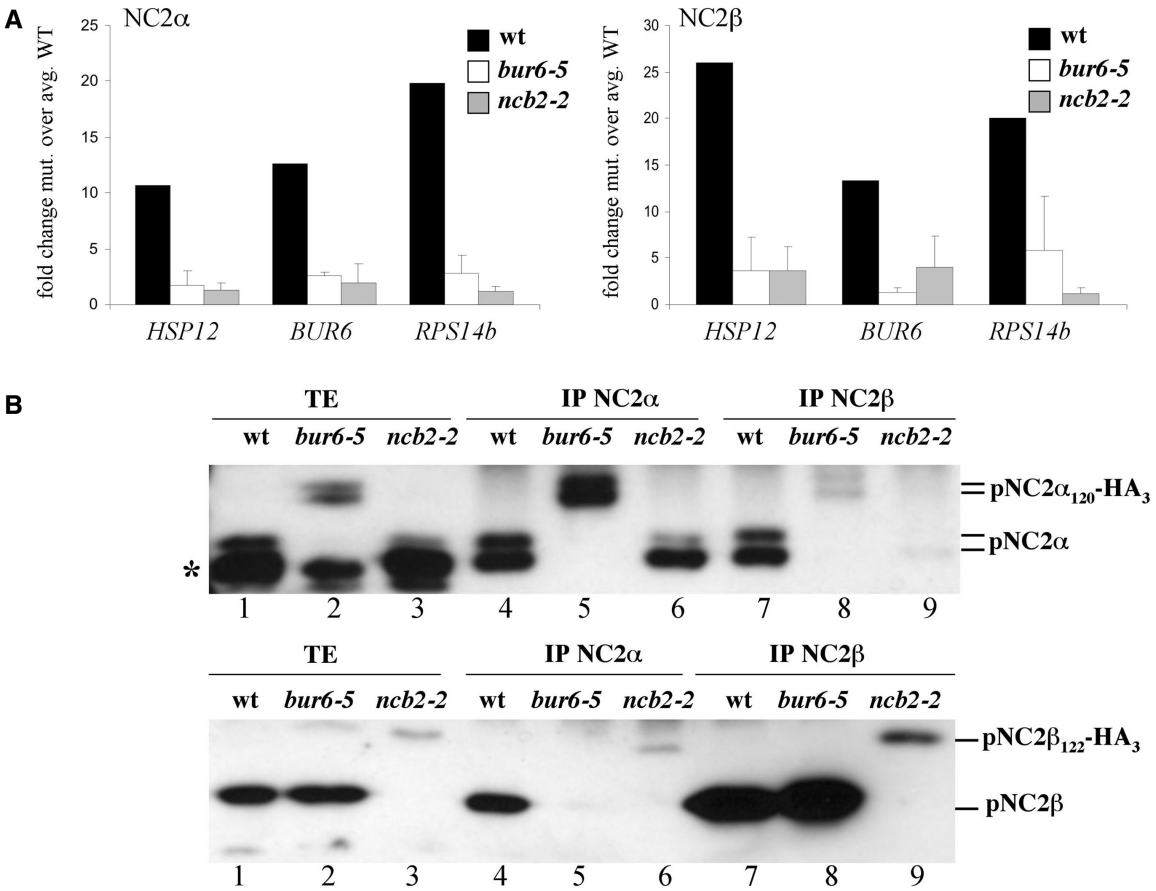

Figure 3. Association of NC2 subunits with DNA in the *bur6-5* and *ncb2-2* mutants. (**A**) Wild-type or mutant cells as indicated were grown in high glucose for 24 h, cross-linked and cell extracts were incubated with antibodies against NC2α or NC2β. After immunoprecipitation and purification of nucleic acids in the immunoprecipitates, the amount of the indicated promoters (*HSP12*, *BUR6* or *RPS14b*) in the precipitates was evaluated by real-time PCR, and referred to the input extract. The experiments were performed three times independently and the results are presented as the fold-change in the mutant over the average wild-type. (**B**) Exponentially growing cells from the strains indicated above the panels were lyzed for total protein extract (TE) preparation. The extracts were incubated with antibodies against NC2α (IP NC2α) or N2β (IP NC2β) followed by protein G Sepharose, and the total extract or immunoprecipitates were analyzed by western blot with polyclonal NC2α (upper panel) or NC2β (lower panel) antibodies as indicated. All samples tested with one antibody were run on the same gel and exposed together for the same length of time. The position of the proteins encoded by *bur6-5* and *ncb2-2*, as well as the proteins encoded by the wild-type genes are indicated on the right of the panels, and on the left, asterisk indicates a band cross-reacting with NC2α antibodies and which co-migrates with one form of NC2α.

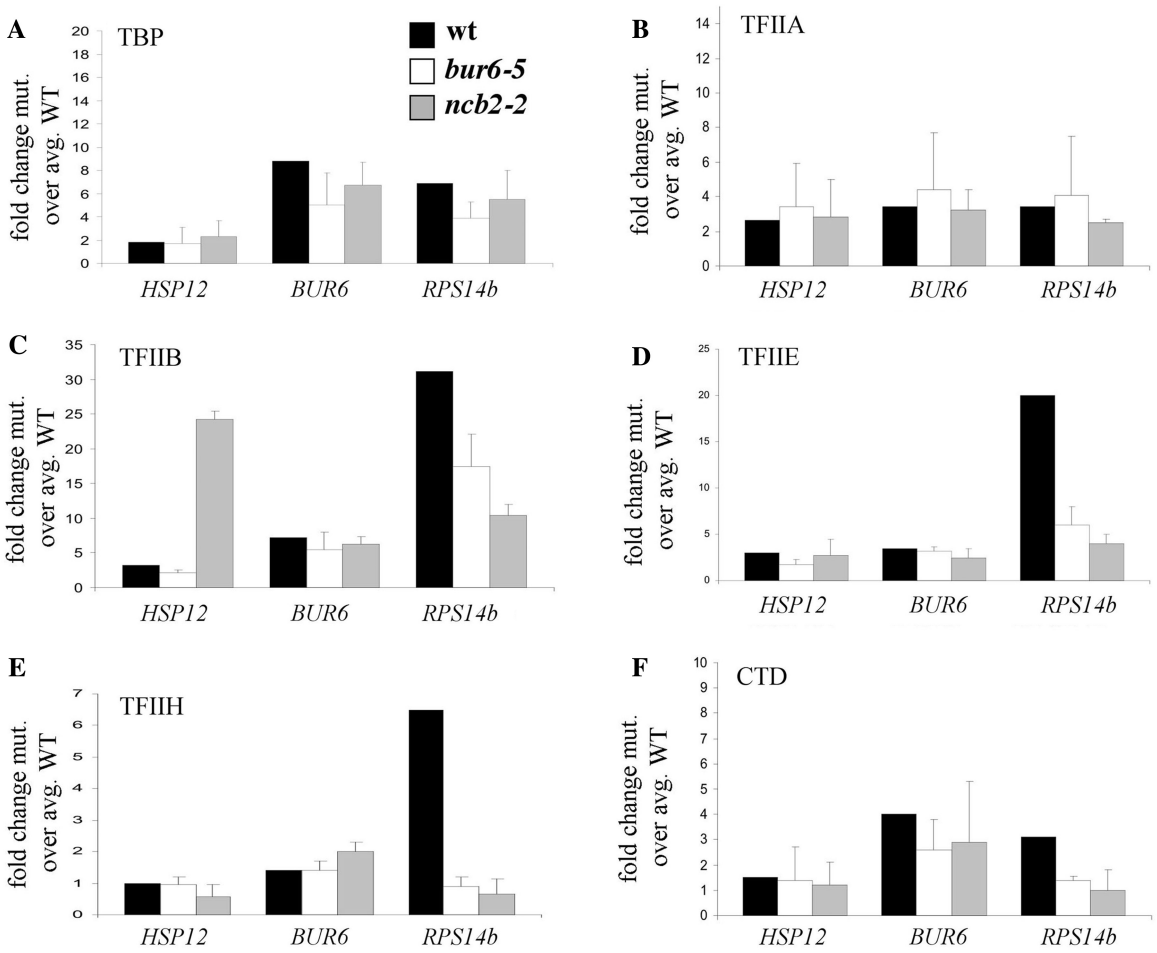

Figure 4. Analysis of PIC recruitment in wt, *bur6-5* and *ncb2-2* cells by ChIP. (**A**) Wild-type or mutant cells as indicated were grown in high glucose for 24 h, cross-linked and cell extracts were incubated with antibodies against TBP. After immunoprecipitation, and purification of nucleic acids in the immunoprecipitates, the amount of the indicated promoters (*HSP12*, *BUR6* or *RPS14b*) in the precipitates was evaluated by real-time PCR and referred to the input extract. The experiments were performed three times independently and the results are expressed as in Figure 3A. (**B**) The same procedure was used to follow TFIIA recruitment with polyclonal antibodies, against Toa1p or with monoclonal antibodies against MYC to follow (**C**) TFIIB using wild-type or mutant strains expressing MYC-tagged Sua7p, (**D**) TFIIE using wild-type or mutant strains expressing MYC-tagged Tfa1p, (**E**) or TFIIH using wild-type or mutant strains expressing MYC-tagged Tfb3p and finally (**F**) monoclonal antibodies directed against the CTD to follow RNA polymerase II.

change in TBP recruitment was observed in the mutants. On *BUR6* and *RPS14b*, there was a slight decrease of TBP in the *bur6-5* mutant, but no significant difference in the *ncb2-2* mutant compared to the wt (Figure 4A). Thus, in cells growing exponentially, the two NC2 mutants do not strongly affect the recruitment of TBP to promoters, whose activity nevertheless changes in the mutants, be it up or down.

In vitro, the NC2 complex has been shown to block the association of the basal transcription factors TFIIA and TFIIB from associating with DNA-bound TBP, resulting in non-productive TBP–TATA complexes (3). Thus, we next investigated TFIIA recruitment to NC2 target genes in the wt and mutant cells. For this, we used antibodies directed against the large subunit of TFIIA called Toa1p. We found no significant change in Toa1p recruitment in either mutant compared to the wt, suggesting that NC2 does not strongly inhibit the recruitment of TFIIA *in vivo*, as previously described *in vitro* (Figure 4B). To next follow TFIIB presence on promoters, we created strains expressing a MYC-tagged form of TFIIB (Table 1). We observed a very strong increase in TFIIB recruitment to *HSP12* in the *ncb2-2* mutant (about a 7- to 8-fold increase compared to the wt) that correlated well with upregulation of the *HSP12* mRNA in this mutant, but no change in TFIIB association with the *HSP12* promoter was detected in *bur6-5*. For the *BUR6* promoter, no significant variations in TFIIB association in the three strains were observed despite the increased levels of *BUR6* mRNA in both mutants. On the *RPS14b* promoter in contrast, a significant decrease of TFIIB association was observed in both mutants (Figure 4C), demonstrating a correlation, for this gene, between the loss of NC2, the loss of TFIIB on the promoter and the decrease of *RPS14b* mRNA, in the mutants. Next, we investigated the presence of TFIIE and TFIIH by creating strains expressing MYC-tagged versions of the Tfa1p and Tfb3p subunits of TFIIE and TFIIH, respectively (Table 1). No significant change in the

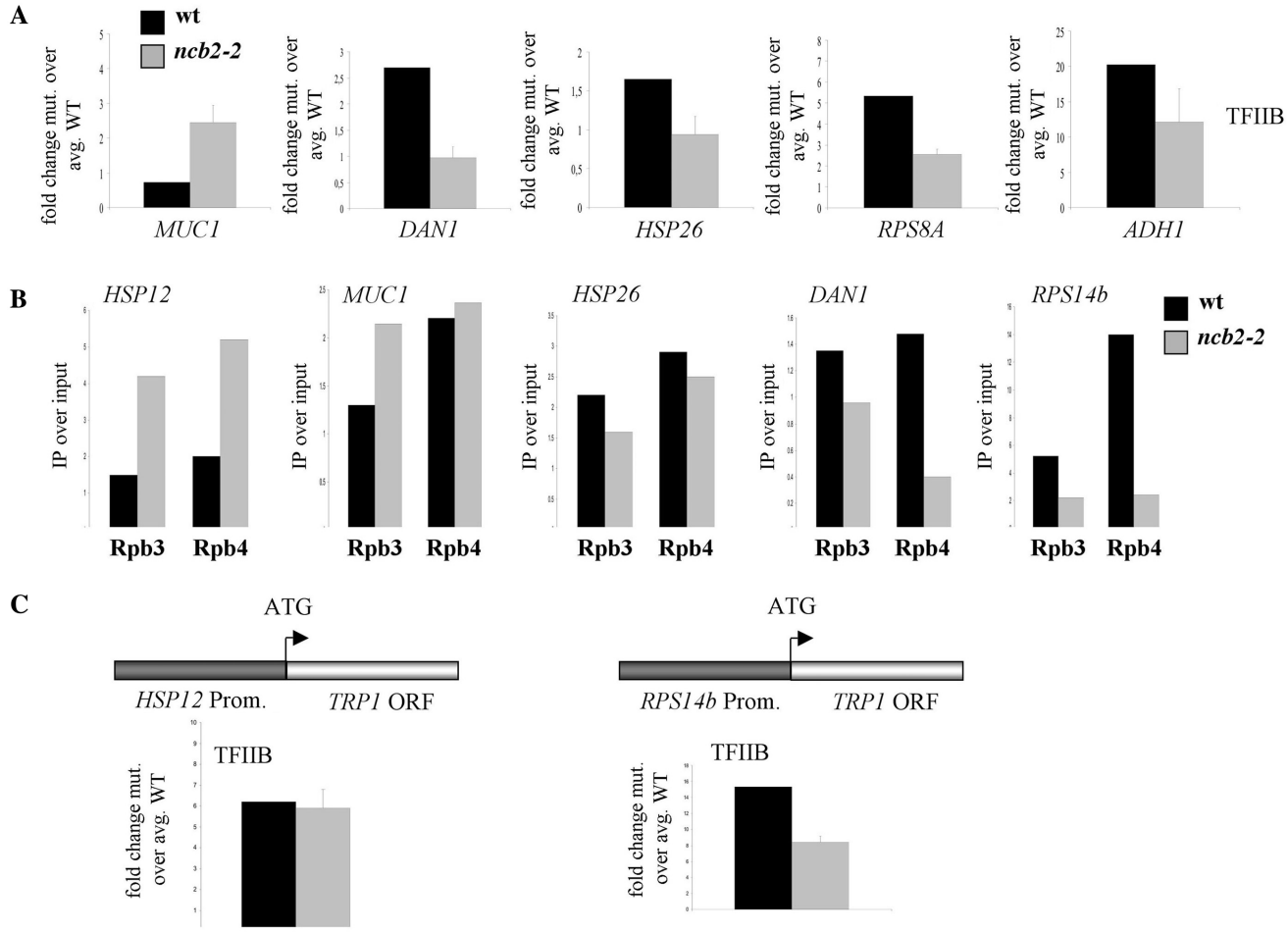

Figure 5. Changes in Pol II and TFIIB recruitment in the *ncb2-2* mutant are gene-specific. (**A**) ChIP experiments were performed to follow TFIIB using wild-type or *ncb2-2* cells expressing MYC-tagged Sua7p. The promoters *MUC1*, *DAN1*, *HSP26 RPS8A* and *ADH1*, were tested and the experiments were performed three times independently and the results expressed as in Figure 4. (**B**) ChIP experiments were performed to follow Pol II using antibodies directed against the Rpb3p or Rpb4p subunits. The *HSP12, MUC1, HSP26, DAN1* and *RPS14b* promoters were tested and referred to the input. The results of one experiment are presented. (**C**) ChIP experiments were performed to follow TFIIB recruitment on the *HSP12* or *RPS14b* promoters when the ORFs of these genes had been replaced by the *TRP1* ORF at the endogenous loci of *HSP12* and *RPS14b*. The experiments were performed three times independently and the results expressed as in Figure 4.

presence of either factor was measured between NC2 mutants and the wt, on either *HSP12* or *BUR6*, but a drastic decrease of both factors on *RPS14b* was observed in the mutants compared to the wt (Figure 4D and E). Finally, to determine how the described changes in association of GTFs might affect transcription, we investigated the presence of RNA polymerase II (Pol II) on the three genes by using antibodies against its carboxy terminal heptad repeat (CTD). There was no significant change in the presence of Pol II on either *HSP12* or *BUR6* in the mutants compared to the wt, but in contrast a clear reduction of Pol II on *RPS14b* in both mutants was observed (Figure 4F).

The differential recruitment of TFIIB in NC2 mutants is highly promoter specific

Since changes in TFIIB association with promoters of *HSP12* and *RPS14b* correlated with activation and repression of these genes in NC2 mutants, we extended our investigation to additional targets genes. For genes upregulated in the *ncb2-2* mutant, a small but significant increase of TFIIB was additionally observed only on *MUC1*, but neither on *DAN1* nor on *HSP26*, where instead we observed a decrease of TFIIB, as we did on all highly expressed genes that we analyzed, such as *RPS8A* or *ADH1* (Figure 5A). Thus, while a reduction of TFIIB association with promoters was observed for many genes in *ncb2-2*, be they repressed, activated or not altered, *in ncb2-2*, a strong increase of TFIIB binding to the promoter in this mutant appeared to be very specific to *HSP12*, with a milder effect on *MUC1*. To confirm this specificity, we examined TFIIB association with the complete chromosome VI using tiling arrays (Affymetrix) by a ChIP-on-ChIP experiment and indeed, the results showed that TFIIB was strongly increased only on the *HSP12* promoter (but not on the *HSP12* ORF) in the *ncb2-2* mutant (data not shown).

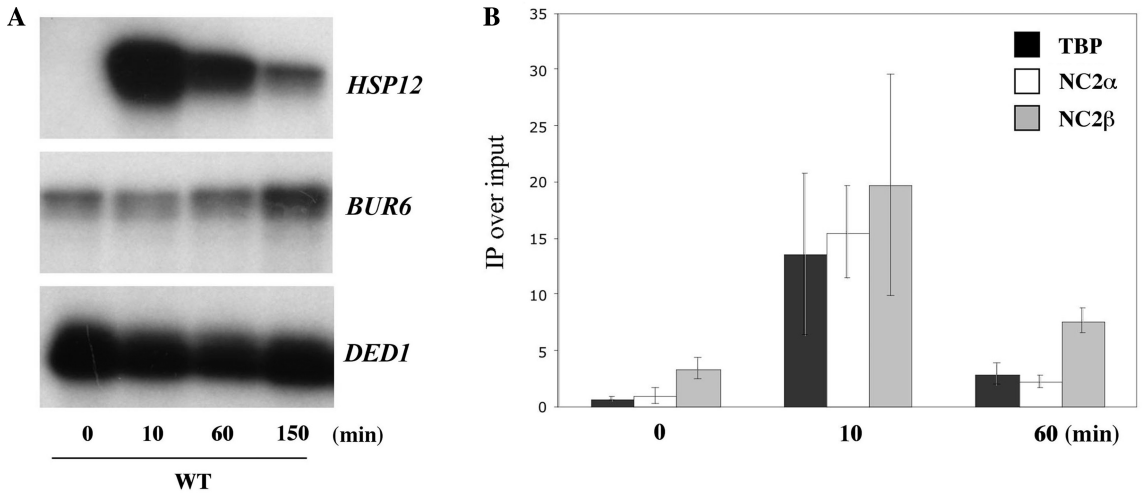

Figure 6. NC2 is massively recruited to the *HSP12* promoter upon heat shock. (**A**) Wild-type cells were grown to exponential phase, and then shifted to 38.5°C for the indicated times. Total cellular RNA was extracted and analyzed by S1 digestion for the levels of *HSP12*, *BUR6* and *DED1* as indicated. (**B**) The same cells were cross-linked with formaldehyde prior to total extract preparation for TBP, NC2α and NC2β immunoprecipitation. The presence of the *HSP12* promoter in the immunoprecipitates was evaluated by real-time PCR and expressed relative to input DNA. The results of three independent experiments are presented.

We next re-examined the recruitment of Pol II, by investigating several NC2 target genes and using antibodies directed against the Rpb3p and Rpb4p subunits of the polymerase. Increases of both Pol II subunits were measured on *HSP12* and to a lesser extent on *MUC1* (Figure 5B), whereas in contrast decreases were measured on *HSP26*, *DAN1* and dramatically on *RPS14b*, in the mutant (Figure 5B). Thus, changes in Pol II association with promoters correlate quite well with changes in TFIIB recruitment in *ncb2-2*, but only partially with changes in gene expression. Indeed, for some genes such as *DAN1* and *HSP26*, changes in association of TFIIB and Pol II are surprisingly opposite to changes in expression.

Finally, to determine the elements driving the differential effects observed on TFIIB recruitment in NC2 mutants depending upon the gene, we generated strains in which the *HSP12* or *RPS14B* ORFs were substituted with an unrelated ORF (see Materials and Methods section). Interestingly, we found that recruitment of TFIIB to the promoter of *HSP12* was similar in wt and *ncb2-2* mutant cells under these conditions (Figure 5C). Thus, the *HSP12* ORF is required to observe NC2 regulation of TFIIB association with its promoter. In contrast, regulation of the presence of TFIIB on the *RPS14b* promoter by NC2 was observed independently upon the ORF immediately downstream (Figure 5C).

NC2 is important for *HSP12* upregulation in response to heat shock

Our results show that in both NC2 mutant strains, there is a global loss of NC2 associated with DNA, but nevertheless relatively few alterations in gene expression, and little effect on cell growth. This raises the question of the excess of NC2 on DNA. To address this issue, we concentrated on *HSP12*, since our present results are compatible with a model in which NC2, which is a positive factor for the formation of the PIC on many genes such as *RPS14b*, is nevertheless a repressor of the PIC for this gene under normal growth conditions. However, a previous report suggests that NC2α is recruited to heat-shock genes along with TBP upon heat shock (12). We thus investigated the role of NC2 on *HSP12* during heat shock. First, a heat-shock time course revealed that *HSP12* is massively induced after 10 min at 38.5°C under our experimental conditions, with a significant drop after 1 h already (Figure 6A). By ChIP experiments, we found that both NC2 subunits were transiently recruited together with TBP within 10 min of heat shock and reduced at the promoter already after 1 h (see Figure 6B). These results suggest a possible importance of the NC2 heterodimer for transcriptional activation of *HSP12* upon heat shock.

This finding led us to investigate the impact of the C-terminal mutations of NC2 on *HSP12* activation after heat stress. We first analyzed the induction of *HSP12* mRNA in wt and mutant strains, and we observed that while *HSP12* is de-repressed in *ncb2-2* under non-stressed conditions, it is also less well activated upon heat shock compared to wt cells. A reduced efficiency of *HSP12* activation upon heat shock was even more pronounced in *bur6-5* (Figure 7A). A recent study demonstrated a strong depletion of histone H3 on the *HSP12* promoter a few minutes after heat shock (20). Here, we found the same clear depletion of histone H3 in all three strains analyzed (Figure 7B) demonstrating that inefficient activation of *HSP12* in the mutants was not due to poor chromatin clearance. We thus investigated the recruitment of both TBP and Pol II by ChIP experiments as before. While a strong induction of both TBP and CTD association with the *HSP12* promoter was observed in wt cells after 10 min, no such striking induction was observed in either NC2 mutant (Figure 7C).

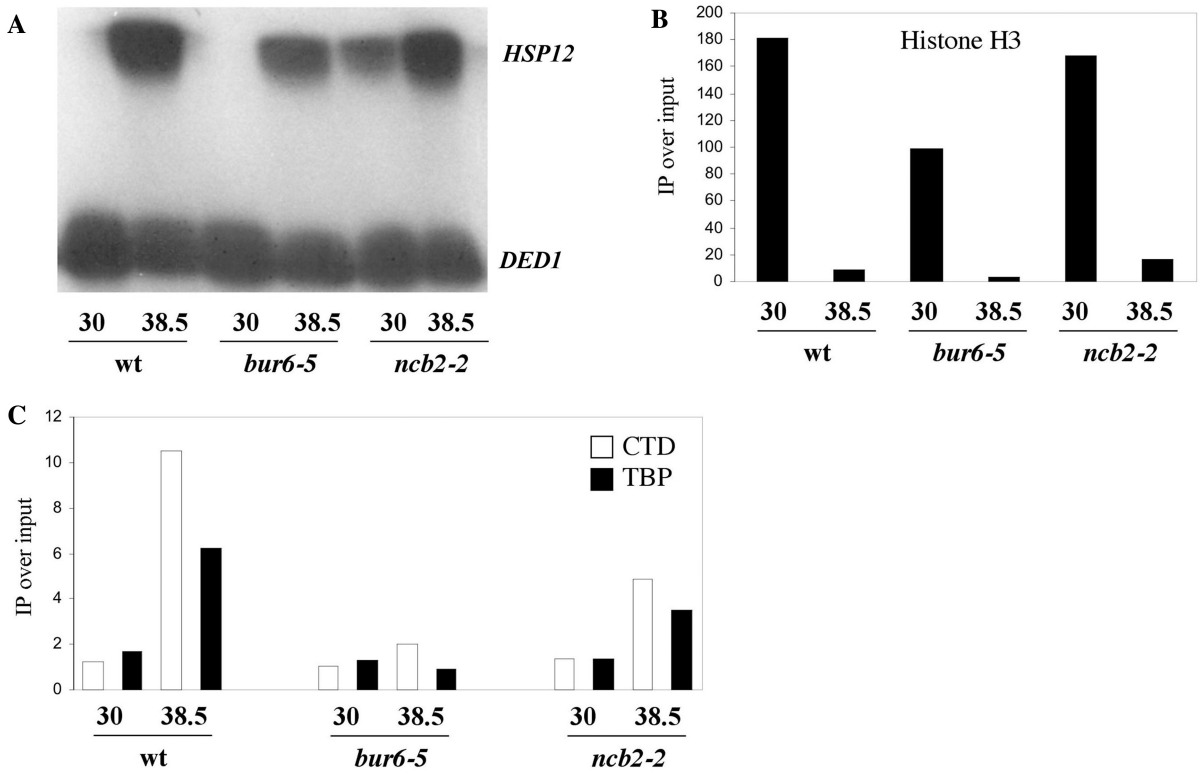

Figure 7. Mutation of either NC2 subunit leads to non-optimal *HSP12* induction upon heat shock. (**A**) Wild-type and mutant cells were grown to exponential phase, and then shifted or not to 38.5°C as indicated for 10 min. Total cellular RNA was extracted and analyzed for the levels of *HSP12* and *DED1* as indicated. Quantification of the blot reveals an induction of more than 850-fold of *HSP12* relative to *DED1* upon heat shock for the wild-type, more than 300-fold for *bur6-5* and 10-fold for *ncb2-2*. (**B**) Wild-type or mutant cells as indicated were grown to exponential phase, and then shifted or not to 38.5°C as indicated for 10 min, cross-linked and ChIP were performed with antibodies against histone H3. (**C**) Wild-type or mutant cells as indicated were grown to exponential phase, and then shifted or not to 38.5°C as indicated for 10 min, cross-linked and ChIP experiments were performed to follow TBP and the CTD with the same antibodies used in the experiments presented in Figure 4.

DISCUSSION

Study of NC2α and NC2β C-terminal deletion mutants reveals the importance of the NC2 heterodimer

In this work, to further our understanding of the NC2 transcriptional co-factor, we studied *S. cerevisiae* cells expressing C-terminally truncated forms of either NC2 subunit. A rather small number of genes, including both *BUR6* (Figure 2) and *NCB2* (data not shown), are affected in these mutants during normal growth, suggesting that NC2 auto-regulates its expression. We find that the two subunits act mostly together in exponential phase. Several results strongly support this hypothesis. First, our microarray results show a significant overlap between genes affected in either NC2 subunit mutant. Second, ChIP experiments show that altering one subunit strongly impairs the recruitment of the second subunit to all DNA sequences tested.

This finding contrasts with our conclusion in a recent report suggesting that NC2α and β might act independently of each other because they do not co-purify (14). Our present results do not exclude the possibility of independent functions for the NC2 subunits, since there are genes deregulated in one mutant and not in the other. However, they also reveal that the NC2 heterodimer is not limiting in cells growing exponentially since *bur6-5* cells are wt for growth despite very much reduced DNA-bound heterodimeric NC2. In the previous study, we used cells expressing tagged versions of the NC2 subunits that express reduced levels of the NC2 heterodimer, which escaped detection, but supported wt growth (our unpublished data). Interestingly, *ncb2-2* cells express similar low levels of the NC2 heterodimer as *bur6-5* cells, but in contrast to *bur6-5* cells, they display a slow growth phenotype. Thus, normal cell growth in high glucose does seem to require the C-terminal domain of NC2β, at least under limiting amounts of the NC2 heterodimer.

PIC assembly is differently affected by NC2 mutants

We addressed the role of the domains C-terminal to the histone-fold motif (HFM) of both NC2α and NC2β in PIC assembly on target promoters *in vivo*. Indeed, structural studies suggest that the C-terminal region of NC2β inhibits TFIIB recruitment to TBP (8), whereas other studies have shown that the C-terminal region of human NC2α supports TBP binding to DNA (16) and interacts with BTAF1 (15). In yeast, sequences besides the HFM of NC2α are dispensable for growth (21). A functional importance for regions besides the HFM have been demonstrated in several proteins, such as the mammalian NF-Y trimeric transcription factor, where stretches at the N- and C-termini of the HFM influence

DNA binding (22) and Taf11p, where the region N-terminal to the HFM contributes to interaction with TFIIA (23). In our study, we observe that the primary consequence of deleting the C-terminal regions of either NC2 subunit is a reduction of the NC2 heterodimer. Thus, mutant phenotypes common to both NC2 mutants reveal roles of the NC2 heterodimer, whereas roles for the C-terminal domains can be inferred from mutant phenotypes specific to one of the NC2 mutants only. Obviously, there may be additional roles of NC2 that have not been revealed in our study of these two specific mutants.

First, concerning TBP, we observed a modest decrease of TBP binding to promoters such as *BUR6* or *RPS14b* for the NC2α mutant only. From this, we conclude that NC2 is not generally contributing to, or limiting for, the association of TBP to promoters. However, the C-terminal domain of NC2α might provide some support for TBP binding to certain promoters, at least under limiting NC2 levels.

For the early recruited factor TFIIA, no significant changes in either mutant on any promoter studied, was identified. Thus, NC2 is not limiting, and no role for the C-terminal regions of both subunits was found, either to support TFIIA association or to counteract TFIIA association, with promoters. This finding surprisingly does not support a previous model suggesting that the essential function of NC2 is to counteract TFIIA (7).

The analysis of another early factor in PIC formation, TFIIB, revealed unexpectedly, that for many genes tested, mutation of either NC2 subunit led to its reduction on promoters. In contrast, out of all the genes tested, only two displayed an increase of TFIIB on their promoter, namely *HSP12* and *MUC1*, and this only in the *ncb2-2* mutant. This result does not support the model provided by *in vitro* studies and the crystal structure that NC2 and the C-terminal region of NC2β in particular, act as a global repressor of transcription by inhibiting recruitment of TFIIB. Rather, it appears that NC2 regulates TFIIB association with promoters in a highly gene-specific and dual manner. A positive role of NC2 on PIC formation is supported by previous biochemical studies with both *drosophila* NC2 (11) and yeast NC2 (6).

The dominant role of NC2 on TFIIB *in vivo* appears to be a positive role, and this seems to be determined by the promoters of the target genes, as we determined for one such gene, *RPS14b*. In particular, TFIIB association with all strongly expressed genes that we looked at requires NC2, in accordance with recent reports suggesting that NC2 is generally activating ribosomal protein (RP) genes (24), but NC2 also has a positive effect on TFIIB recruitment to some lowly expressed genes such as *HSP26* or *DAN1*. Factors which assemble after TFIIB in the PIC, namely TFIIE, TFIIH as well as RNA polymerase II, generally also decrease in NC2 mutants. However, mRNA levels do not always correlate with these effects, since some mRNAs decrease consistently, such as *RPS14b*, some do not change such as *ADH1* and some even increase, such as *DAN1* or *HSP26*. One possibility is that GTFs are limiting for *RPS14b*, but not limiting for genes such as *ADH1*, *DAN1* or *HSP26*. The latter two mRNAs might additionally be indirectly increased by some post-transcriptional mechanism. This might also be the case for *BUR6*, for which we found no changes in the transcription machinery at the promoter that could correlate with the increased levels of the mRNA in the NC2 mutants.

In contrast, the identified negative role of NC2, and in particular of the C-terminal domain of NC2β, on TFIIB association with promoters is very restricted. Indeed, *HSP12* was the only target for such an effect identified on the entire chromosome VI by ChIP-on-ChIP experiments (data not shown). The *HSP12* ORF, unique in the genome, is required for this regulation, explaining why the effect is so restricted. Surprisingly, the increased association of TFIIB with *HSP12* in the *ncb2-2* mutant correlates with a weak increase of Pol II, but not with any increase of either TFIIE or TFIIH. Thus, TFIIB on this promoter might be promoting transcription mostly at a step subsequent to polymerase binding in the mutant, leading for instance, to more productive elongation from a stalled or inefficient polymerase, taking as a model the block to elongation that has been described for mammalian or *Drosophila* heat-shock genes (25). Alternatively, the increased presence of TFIIB might accelerate re-initiation, play some role in efficient mRNA export or even not play an active role, but reflect the disappearance of an antisense transcript that prevents accumulation of a sense transcript in wt cells. This latter hypothesis integrates our observation that this regulation requires the *HSP12* ORF, and a previous study revealing the presence of an antisense transcript for *HSP12* (26). Furthermore, such a model could extend to other genes whose expression increases in NC2 mutants, such as *DAN1*, *HSP26* and *BUR6* for which we found no increase of tested components of the PIC at the promoter.

Efficient *HSP12* transcriptional activation requires intact NC2

Our study suggests that NC2 is bound in excess on many promoters in cells growing exponentially. It appears however, that for genes such as *HSP12*, this might be important in order to allow activation to high levels in response to the appropriate stimulus. This is consistent with a previous study that has suggested an important role of NC2 in the stress response (12) and our finding that the association of both NC2 subunits to the *HSP12* promoter is transiently increased together with TBP, in response to heat shock. Furthermore, efficient recruitment of TBP and transcriptional activation upon heat shock requires the presence of sufficient NC2, and the NC2 mutants that have reduced NC2 heterodimer are temperature sensitive. It has been shown that NC2 can play a role in nucleosome sliding and assembly (17), and it is known that histones are depleted from heat-shock promoters upon heat shock (27). However, we found that histone H3 is correctly depleted from *HSP12* upon heat shock in the NC2 mutants, suggesting that the effect of NC2 on TBP is not indirectly due to an effect on histone depletion.

Taken together, our results suggest that NC2 plays a positive role on genes that are highly expressed, but through different GTFs in unstressed (TFIIB) or stressed

(TBP) cells. In this regard, it is interesting to note that NC2 and Mot1p, another regulator of TBP, have been described to have common target genes (28), were identified in at least two similar genetic screens for transcriptional repressors (21), and that Mot1p, like NC2, is transiently recruited to heat-shock genes upon heat shock (29). However, whereas mutation of NC2 reduces activation of heat-shock genes in response to heat shock, mutation of *MOT1* leads to increased expression of heat-shock mRNA levels (29) and to an increased presence of NC2 at promoters (28). Therefore an inter-play between NC2 and Mot1p might determine the importance of NC2 for the association of TBP with DNA, particularly upon heat shock.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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