The NC2 α and β subunits play different roles in vivo

Sandrine Creton,¹ Jesper Q. Svejstrup,² and Martine A. Collart^{1,3}

¹Department Biochimie Médicale, CMU, Geneva 4, Switzerland; ²Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK

NC2 is a heterodimeric regulator of transcription that plays both positive and negative roles in vivo. Here we show that the α and β subunits of yeast NC2 are not always associated in a tight complex. Rather, their association is regulated, in particular by glucose depletion. Indeed, stable NC2 α/β complexes can only be purified from cells after the diauxic shift when glucose has been depleted from the growth medium. In vivo, the presence of NC2 α , but not NC2 β , at promoters generally correlates with the presence of TBP and transcriptional activity. In contrast, increased presence of NC2 β relative to TBP correlates with transcriptional repression. NC2 is regulated by phosphorylation. We found that mutation of genes encoding casein kinase II (CKII) subunits as well as potential CKII phosphorylation sites in NC2 α and β affected gene repression. Interestingly, NC2-dependent repression in the phosphorylation site mutants was only perturbed in high glucose when NC2 β and NC2 α are not associated, but not after the diauxic shift when NC2 α and β form stable complexes. Thus, the separation of NC2 α and β function indicated by these mutants also supports the existence of multiple NC2 complexes with different functions in transcription.

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Assembly of a functional preinitiation complex on core promoters of protein coding genes in eukaryotic cells requires RNA polymerase II and general transcription factors, as well as chromatin remodeling activities. Once the chromatin has been remodeled, the initial and rate limiting event is the recruitment of the TATA-binding protein (TBP) to the core promoter (Klein and Struhl 1994), which then nucleates the assembly of the rest of the complex, initially by the recruitment of TFIIA and TFIIB (Nikolov et al. 1995; Geiger et al. 1996; Orphanides et al. 1996; Tan et al. 1996). The best characterized form of TBP recruited to core promoters is TFIID, which consists of TBP and multiple TAF_{II}s (TBP-associated factors; Verrijzer and Tjian 1996). However, it is generally thought that other forms of TBP also play important roles in transcriptional regulation (Kuras et al. 2000; Li et al. 2000). Besides the TAF_{II}s, examples of other TBP-associating factors include the Ccr4-Not complex (Collart and Struhl 1994; Lee et al. 1998; Liu et al. 1998), the Mot1 ATPase (Timmers et al. 1992; Auble et al. 1994; Van der Knaap et al. 1997; Chicca et al. 1998) and the NC2 histone-fold heterodimer (Bur6p-Ydr1p, α - β , or Drap1-Dr1; Meisterernst and Roeder 1991; Inostroza et al. 1992; Goppelt et al. 1996; Cang et al. 1999).

It has been suggested that these factors are global repressors of transcription that also play positive roles in vivo (Collart 1996; Madison and Winston 1997; Prelich 1997; Liu et al. 1998).

NC2 was initially purified from human cell extracts as an activity that inhibits basal TATA-dependent transcription in vitro (Meisterernst and Roeder 1991; Inostroza et al. 1992). It was subsequently identified as an essential factor in yeast [encoded by the *BUR6* (NC2 α /Bur6p) and *NCB2* (NC2 β /Ydr1p) genes] that is both conserved and functionally interchangeable between yeast and human (Goppelt and Meisterernst 1996; Kim et al. 1997; Lemaire et al. 2000). Many experiments define NC2 as a transcriptional repressor. First, after its initial identification, experiments in vitro showed that NC2 could exert variable extents of repression on transcription, depending upon the core promoter (Kim et al. 1996; Willy et al. 2000). NC2 associates with promoter-bound TBP, thereby preventing the recruitment of TFIIA and TFIIB to the promoter (Goppelt et al. 1996). Subsequent studies in yeast identified a mutation in the largest subunit of TFIIA as a suppressor of the essential role for NC2 providing in vivo support for the results of the in vitro studies (Xie et al. 2000). Furthermore, mutations in TBP that prevent the interaction with NC2 were isolated and found to locate near the surfaces of TBP that also mediate association with TFIIB (Cang et al. 1999). Finally, a recent crystal structure of NC2 recognizing the TBP-DNA transcription complex nicely demonstrates

³Corresponding author.

E-MAIL martine.collart@medecine.unige.ch; FAX 41-22-702-55-02

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how NC2 binding might preclude recruitment of TFIIB and TFIIA to a preformed TBP–DNA complex (Kamada et al. 2001).

In addition to its well-characterized role as a repressor, several experiments suggest that NC2 might also play positive roles in transcription. First, yeast cells expressing a mutant form of the α subunit of NC2, and cells in which NC2 α is depleted, as well as cells that have altered NC2 β activity, display both increased and decreased transcript levels *in vivo*, depending upon the gene studied (Prelich 1997; Lemaire et al. 2000; Geisberg et al. 2001). Second, the *Drosophila* homolog of NC2 was isolated from extracts as an activity capable of activating transcription from promoters that carry an element conserved in *Drosophila* and humans, but not in yeast, called the DPE (downstream promoter element; Willy et al. 2000). Finally, a study in yeast showed that the association of the α subunit of NC2 to promoters generally correlates with transcriptional activity and with occupancy by general transcription factors (Geisberg et al. 2001). Taken together, these studies suggest that NC2 might be an activator as well as a repressor. However, the molecular mechanisms that enable such a dual function are unknown.

We have previously shown that both subunits of NC2 are required for transcription of the yeast *HIS3* gene from its TATA-less promoter in exponentially growing cells, as well as for repression of the same gene from its TATA promoter at the diauxic shift (Lemaire et al. 2000). We used these opposite effects of NC2 on *HIS3* as a starting point to dissect how NC2 may act both as a transcriptional repressor and a transcriptional activator. Here we report that the two NC2 subunits are not tightly associated in exponentially growing cells, but are able to form a stable complex upon glucose depletion. Furthermore, whereas the α subunit of NC2 can be found at promoters together with TBP in correlation with transcriptional activity, an increased ratio of NC2 β to NC2 α and TBP at promoters correlates with transcriptional repression. The existence of separate forms of the NC2 α and β subunits, taken together with the different association of the α and β subunits of NC2 with promoters, allows us to propose that the two subunits play distinct roles *in vivo*. This work provides a conceptual framework to explain how mutations in transcription factor genes can have pleiotropic effects, and suggests a molecular mechanism that can enable both positive and negative functions for regulatory factors *in vivo*.

Results

Different forms of NC2 α and β subunits in cells before and after the diauxic shift

We took a biochemical approach to try and understand the pleiotropic effects of NC2 on transcription. We first analyzed the gel filtration elution profile of the NC2 α and β subunits in yeast cells before and after the diauxic shift (Fig. 1). We found that the β subunit from exponentially growing cells eluted in fractions 23–27 correspond-

Figure 1. Different forms of NC2 α and β subunits before and after the diauxic shift. Three-hundred microliters of total cell extracts from wild-type cells grown exponentially or to the post-diauxic phase, as indicated, was fractionated by Superose 6 gel filtration. The resulting fractions were analyzed by Western blot analysis with antibodies against NC2 β (A) or α (B), as indicated. The peaks of elution from exponential phase cells are indicated by $\blacklozenge$, whereas novel complexes appearing in the postdiauxic phase are indicated by *. The sizes indicated from the elution of molecular weight markers (Bio-Rad) are shown above the fractions.

ing to a size of ~ 150 kD. After the diauxic shift, however, NC2 β eluted with a much broader profile, in fractions 19–31, with new forms found in fractions 19–21, and a broadening of the peak in fraction 25 towards smaller sizes (Fig. 1A). The α subunit of NC2 eluted with a similar profile, mainly in fractions 23–25 from cells growing exponentially, and in fractions 19–27 in cells after the diauxic shift (Fig. 1B).

Taken together, these results indicate that both the α and the β subunits of NC2 are associated in complexes of ~ 150 kD in exponentially growing cells, and additionally become associated in larger complexes of ~ 400 kD after the diauxic shift. It is interesting to note that both subunits also additionally elute as species smaller than 150 kD after the diauxic shift.

Purification of the β subunit of NC2

To better understand the biochemical behavior of the NC2 complex, we set out to purify the β subunit from extracts made from exponentially growing cells and postdiauxic phase cells, respectively. For this purpose, a strain was constructed in which the β subunit carried a double affinity tag at its C terminus (see Table 1; Winkler et al. 2001). We then purified the subunit through several chromatographic steps including Bio-Rex 70 chromatography, HA-affinity chromatography, and finally nickel–agarose chromatography. We used low salt conditions in order not to disrupt putative fragile complexes. Surprisingly, whereas NC2 β from exponentially growing cells bound well to the affinity columns, the epitope tag was not accessible on NC2 β from extracts of postdiauxic phase cells (Fig. 2A). Thus, NC2 β was purified from exponentially growing cells and coomassie staining revealed a single protein that was confirmed by mass spectroscopy to be the β subunit of NC2 (Fig. 2B). Significantly, the purified β subunit eluted from a gel filtration column with an elution profile similar to the

Table 1. *Strains*

Strain	Genotype	Reference
MY1	<i>MATa gcn4Δ ura3-52 trp1Δ1 leu2::PET56 gal2</i>	(Hope and Struhk 1986)
MY114	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56</i>	This study
MY1800	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{ADH1}hNCB2-LEU2</i>	(Lemaire et al. 2000)
MY2407	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{ADH1}HA-NCB2-LEU2</i>	This study
MY2493	Isogenic to MY1 except <i>bur6::BUR6-3HA-KanMX4</i>	This study
MY2556	<i>his4-917Δ lys2-173R2 leu2Δ1 ura3-52 suc2ΔUAS(-1900/-390) trp1Δ63 spt15Δ102::LEU2 pSPT15-URA3</i>	(Cang et al. 1999)
MY2658	Isogenic to MY2556 except <i>bur6::BUR6-3HA-KanMX4</i>	This study
MY2699	Isogenic to MY1 except <i>MATa ncb2::KanMX4 pP_{NCB2}NCB2-HIS₁₀-HA-TRP1</i>	This study
MY2952	Isogenic to MY1 except <i>MATa bur6::KanMX4 pP_{BUR6}BUR6-HIS₁₀-HA-TRP1</i>	This study
MY3223	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{NCB2}NCB2-URA3</i>	This study
MY3412	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{NCB2}ncb2-1-LEU2</i>	This study
MY3413	Isogenic to MY1 except <i>bur6::KanMX4 + pP_{BUR6}bur6-1-LEU2</i>	This study
MY3351	Isogenic to MY114 except <i>ncb2::KanMX4 + pP_{NCB2}HA-NCB2-LEU2</i>	This study
MY3352	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{NCB2}HA-NCB2-LEU2</i>	This study
MY3357	Isogenic to MY1 except <i>ncb2::KanMX4 + pP_{NCB2}ncb2-2-3HA-LEU2</i>	This study
YDH6	<i>MATa cka1-Δ1::HIS3 cka2-Δ1::TRP1 pCKA2-LEU2</i>	(Hanna et al. 1995)

one observed with the β subunit from crude extracts of cells growing exponentially (Fig. 2C; cf. Fig. 1A), supporting the idea that this is the major, if not only, form of NC2 β found in exponentially growing cells. These results, combined with the fact that NC2 β is a 17-kD protein, suggest that the protein forms homo-oligomers in cells growing exponentially in glucose, rather than being associated in stable complexes with other proteins. In particular, the α subunit did not copurify with the β subunit, as this protein was neither detectable by coomassie staining nor mass spectroscopy analysis of the purified fraction.

Because the C-terminal tag of NC2 β was not accessible in extracts derived from glucose-depleted cells, the purification was repeated from a strain expressing NC2 β with a single epitope (HA) at the N terminus of the protein. In extracts from this strain, the epitope was accessible under all growth conditions tested (see below). Western blot analysis of the partially purified material from this purification confirmed our previous finding that NC2 α does not copurify with NC2 β from extracts made from cells in exponential phase (Fig. 3A). In contrast, NC2 α did copurify with NC2 β from glucose-depleted cells (Fig. 3A), suggesting a de novo association of the two subunits following glucose depletion at the diauxic shift. Analysis of partially purified NC2 β by gel filtration (Fig. 3B) demonstrated that the purified NC2 β had an elution pattern that was similar to NC2 β from crude cell extracts. Furthermore, the copurified α protein from postdiauxic phase cells eluted with a peak in fraction 21, corresponding to a form of NC2 β that was only observed in extracts from cells grown to the postdiauxic phase (see Fig. 1C).

Taken together, these results indicate that in cells growing exponentially in glucose, the NC2 β subunit forms a homo-oligomeric complex of ~100–150 kD, and is not associated in a stable complex with the α subunit. At the diauxic shift, the α subunit then associates with NC2 β to form novel larger complexes.

Purification of the NC2 α subunit

To investigate the biochemical behavior of NC2 from the point of view of NC2 α , epitope-tagged NC2 α was now purified from cells growing exponentially and cells grown to the postdiauxic phase, respectively (Fig. 4A). Western blot analysis of purified NC2 α fractions derived from extracts of glucose-depleted cells indicated the presence of copurifying NC2 β , whereas the β subunit was absent in the purified fraction derived from exponentially growing cells (Fig. 4A). The purified NC2 α fractions were also analyzed by coomassie staining (Fig. 4B). This analysis revealed a number of proteins in addition to NC2 α . Whether these additional proteins are associated in a stable complex with NC2 α or are primarily contaminants remains to be resolved. In any case, the β subunit was clearly detectable by coomassie staining (Fig. 4B) and Western blotting (Fig. 4A) of the purified NC2 α fractions from postdiauxic phase cells, but not in the fractions derived from exponentially growing cells.

Taken together, these results confirm that the α and β subunits of NC2 are not associated in a stable complex in exponentially growing cells, but that they do form stable complexes as glucose is depleted.

The α and β subunits of NC2 are not generally found together at promoters

Because the results presented above suggest that the two subunits of NC2 are not always associated in a stable complex, we investigated their association with promoters in vivo by chromatin immunoprecipitation (ChIP). These experiments were done in situations where the tested genes are well expressed, as well as in a situation in which NC2-dependent repression has previously been described. We chose to look at cells growing exponentially in glucose, and shifted to medium without glucose for up to 3 h to mimic the diauxic shift. Under both these conditions, *ADH1* is well expressed, with a decrease in

Figure 2. Purification of epitope-tagged NC2 β. (A) Total cell extracts from a strain expressing C-terminally epitope-tagged NC2 β (MY2699) grown to exponential or postdiauxic phase, as indicated, were loaded on a HA-affinity column after elution from a Bio-Rex 70 column. The column load (LOAD), flow-through (FT) or eluate (ELUTION) were analyzed by Western blotting using antibodies against NC2 β. (B) The eluate from the final nickel-agarose column was analyzed by 12.5% SDS-PAGE, followed by coomassie staining (upper panel) or Western blot analysis with antibodies against NC2 β (lower panel). Migration of NC2 β is indicated. (C) Fraction 4 from nickel-agarose was subjected to Superose 6 gel filtration and the indicated fractions were analyzed by Western blot analysis with antibodies against NC2 β. The fractions that were indicated by ♦ and * in the equivalent experiment in Figure 1 are indicated for comparison.

expression after glucose depletion. In contrast, *HSP104* is activated by glucose depletion (Fig. 5C), whereas *HIS3* transcription becomes repressed in an NC2-dependent manner (Lemaire et al. 2000). We used strains that expressed tagged versions of the α and β subunits of NC2 and verified that the two subunits could be immunoprecipitated both from extracts of cells growing exponentially in the presence of glucose, and from cells depleted for glucose (Fig. 5A; data not shown).

As previously described by others (Geisberg et al. 2001), we found that occupancy of the tested promoters by NC2 α alone generally correlated with occupancy by TBP. Thus, like TBP density, NC2 α density on the *HSP104* promoter increased under conditions where the *HSP104* gene was transcriptionally induced, whereas it was constantly high on the *ADH1* promoter (Fig. 5C). Surprisingly, in both cases, TBP and NC2 α remained

associated with the promoters after transcript levels decreased. Likewise, NC2 α was present in exponentially growing cells on the active *HIS3* promoters (Fig. 5B). In contrast, no association of the NC2 β subunit with the same promoters under conditions where they were active could be detected (Fig. 5B,C). Significantly, both NC2 β and NC2 α could be detected at the *HIS3* promoters when this gene was repressed (Fig. 5B).

These results indicate that NC2 α and NC2 β are not generally together at promoters. In contrast to NC2 α, NC2 β association at promoters does not correlate with association of TBP, at least at our test genes under the conditions studied. The presence of NC2 β at a promoter does, however, correlate with transcriptional repression, and occurs under growth conditions in which the NC2 α and NC2 β subunits are associated. This suggests that the association of NC2 α with NC2 β could transform this protein into a repressor.

Repression by NC2 correlates with increased presence of NC2 β relative to NC2 α and TBP at promoters

To extend these studies, we set out to identify additional genes that might be regulated by NC2 and in particular

Figure 3. Purification of NC2 β epitope tagged at the N terminus. (A) Total cell extracts from a strain expressing N-terminally epitope-tagged NC2 β (MY2407) grown to exponential or postdiauxic phase as indicated, were purified via Bio-Rex 70, DEAE Sepharose, 12CA5-affinity, and nickel-agarose chromatography. The indicated fractions from the final nickel-agarose column were analyzed by Western blotting with antibodies against NC2 β or α as indicated. (B) The equivalent of 30 μL eluate from the HA-affinity column was subjected to Superose 6 gel filtration. The resulting fractions were analyzed by Western blotting with antibodies against NC2 β or α, as indicated. The fractions that were indicated by ♦ and * in the equivalent experiment in Figure 1 are indicated for comparison.

Figure 4. Purification of epitope-tagged NC2 α . (A) Total cell extracts from cells expressing C-terminally tagged NC2 α (MY2952) grown to exponential (E) or postdiauxic (PD) phase, as indicated, were purified via Bio-Rex 70, DEAE Sepharose, HA-affinity resin and nickel-agarose. Purified fractions from nickel-agarose were analyzed by Western blotting with antibodies against NC2 α and β , as indicated. (B) Purified NC2 α (from the nickel eluate) was loaded on a gradient gel (NuPAGE, Invitrogen) and the gel was analyzed by coomassie staining. Molecular weight markers are indicated on the left (in kD).

repressed by NC2 at the diauxic shift. We compared transcript levels in a wild-type strain and in cells carrying a centromeric plasmid expressing human NC2 β to replace the chromosomal disruption of *NCB2*. These cells are viable, but mutant for several NC2 β functions as previously reported (Lemaire et al. 2000). We found that many genes repressed at the diauxic shift in wild-type cells were less well repressed in the mutant strain. These include *RPS8A*, *CLN2*, *PYK1*, *DBP2*, *ACT1*, *HIS4*, *ADH1*, and to a lesser extent *PGK1*, as shown on Figure 6A. Genes that were not under the same control included *CDC31*, which was somewhat more expressed in mutant cells than in wild-type cells during exponential growth, but was equally well repressed upon glucose depletion (Fig. 6A). In yet another example, *AGA2* was not down-regulated at the diauxic shift in wild-type or mutant cells (Fig. 6A). Finally, we found that appropriate repression of *HSP12* in exponentially growing cells and activation of *HSP12* at the diauxic shift was also dependent on NC2 β (Fig. 6A).

We next used ChIP experiments to try and correlate the presence of NC2 α and NC2 β at promoters with expression of the studied genes (Table 2). We found that there was generally very little change in the NC2 α /TBP ratio at promoters between cells growing in presence or absence of glucose. This is in agreement with previous results obtained by others showing that the presence of

NC2 α at promoters generally follows that of TBP and transcriptional activity (Geisberg et al. 2001). The only exception was the *DBP2* promoter where the amount of NC2 α relative to TBP dropped dramatically after the diauxic shift (Table 2). In contrast, the NC2 β /TBP ratio invariably increased upon glucose depletion at the promoters that were repressed in an NC2- β -dependent manner (see Fig. 6A for expression data; Table 2). This was particularly striking for the *RPS* genes. Significantly, at the *AGA2* promoter, which was not repressed upon glucose depletion, there was no increase in the ratio of NC2 β to TBP. Finally, for the *HSP12* and *HSP26* promoters, the NC2 β /TBP ratio was three- to fourfold higher in cells growing in glucose compared to cells lacking glucose. This correlated nicely with the observation that *HSP12* was repressed in an NC2- β -dependent manner in exponentially growing cells in glucose (see Fig. 6A). The changes in the relative levels of NC2 β to TBP result in most part from the fact that whereas TBP (and NC2 α) association at promoters changes when transcription is induced or repressed, the amount of NC2 β associated with promoters does not change much (Fig. 6B).

We finally repeated ChIP experiments using a wild-type strain and polyclonal antibodies raised against the β subunit of NC2 or TBP, to be confident about the modulations of the NC2 β /TBP ratio described above (Table 3). We were able to confirm that the NC2 β /TBP ratio was higher at the promoters under conditions where such promoters are repressed in an NC2- β -dependent manner, such as after the diauxic shift for *RPS8A*, and to a lesser extent *CLN2*, and before the diauxic shift for *HSP12*.

Taken together, these results demonstrate that the presence of NC2 α and NC2 β at promoters does not generally correlate, and that the presence of the subunits at promoters changes independently. These results furthermore show that the amount of NC2 β relative to TBP at promoters increases upon repression if the gene in question is repressed in an NC2- β -dependent manner.

Mutant studies lead to a distinction of the different NC2 functions in vivo

The results presented above suggest that the relative presence at promoters of the α and β subunits of NC2 is variable and important for transcriptional regulation by NC2. Together with our observation that NC2 α and β do not always form a stable complex, these results suggest that different functions of NC2 might be mediated by different forms of the factor. Since NC2 has been described to be a phosphoprotein and the phosphorylation status of NC2 is thought to affect its interaction with TBP and DNA (Inostroza et al. 1992; Goppelt et al. 1996), we investigated the role of phosphorylation in the different NC2 functions. For this purpose, we created several mutant alleles, expressing derivatives of either subunit with mutations in different putative phosphorylation sites (Fig. 7A). We also created a truncated version of NC2 β , lacking the C-terminal domain beyond amino acid 122 and carrying instead a triple HA epitope. This mutant is partially defective in repression (Kim et al.

Figure 5. The association of NC2 α with promoters correlates with the presence of TBP at promoters, whereas the presence of both NC2 α and β correlates with transcriptional repression. Wild-type cells (MY1 or MY2556) or cells expressing HA-tagged NC2 α (MY2493 or MY2658) or NC2 β (MY2407 or MY3351) were grown exponentially in glucose-rich medium, washed, and transferred to rich medium lacking glucose for up to 3 h. NC2 α and β were immunoprecipitated with antibodies against HA (A), or were cross-linked for ChIP experiments (B,C), or, finally, total RNA was prepared for S1 analysis of *ADH1* or *HSP104* mRNA levels (C). (A) Equal amounts of total extract (WCE) and supernatant (FT), or last wash (W) and immunoprecipitate (IP) were analyzed by Western blotting with NC2 α and β antibodies, as indicated. (B,C) For ChIP experiments, fixed cells were lysed, and incubated with HA antibody and protein A Sepharose, or TBP antibody and protein G Sepharose, for immunoprecipitation. (B) The presence of *HIS3* or *PHO5* (as a control) promoter DNA in the immunoprecipitate was analyzed by radiolabeled PCR (25 cycles). As a control for the equal presence of the investigated DNAs in the strain with (T) or without (U) the tag, the DNA present in the total extract before immunoprecipitation was also analyzed by PCR (INPUT). (C) Input DNAs were comparable for all strains, and the amount of promoter DNA in the immunoprecipitates was expressed in arbitrary units relative to input DNA. The somewhat lower levels of NC2 α occupancy of the *ADH1* promoter before glucose depletion was not reproducible. Controls (cont) were either immunoprecipitation from untagged strains (for HA) or processing without antibody (for TBP). Fifty micrograms of total cellular RNA from wild-type cells growing with glucose, or 1 and 3 h after glucose depletion, as indicated, was analyzed for *ADH1* and *HSP104* expression. Identical results were obtained with all three strains, and thus only the results for the wild-type strain is shown.

2000). The function of the mutant alleles was studied through the analysis of the regulation of *HSP12* and *RPS8A* in exponential and postdiauxic phase cells. Most of the mutations had little or no effect on the regulation of *HSP12* or *RPS8A* (data not shown). However, in cells expressing NC2_{T64A} α or NC2_{S112A} β , repression of *HSP12* in exponentially growing cells and activation of *HSP12* at the diauxic shift was reduced, whereas repression of *RPS8A* at the diauxic shift was normal (Fig. 7B). In contrast, cells expressing a truncated NC2 β derivative that lacked the C-terminal repression domain were defective in repression of *HSP12* in exponentially growing cells and *RPS8A* at the diauxic shift, but activated *HSP12* normally at the diauxic shift (Fig. 7B).

Threonine 64 in NC2 α and serine 112 in NC2 β are putative phosphorylation sites for casein kinase II (CKII). Thus, to determine whether CKII might be important for NC2 function as previously suggested by studies in vitro (Goppelt et al. 1996), we analyzed regulation of *HSP12* and *RPS8A* in cells lacking *CKA1* (encoding one of the two catalytic subunits of CKII; Chen-Wu et al. 1988). We found that such cells had higher levels of the *HSP12* transcripts in exponentially growing cells and lower levels of *HSP12* at the diauxic shift compared to wild-type cells, but had normal repression of *RPS8A* at the diauxic shift (Fig. 7C). Interestingly, we observed a similar effect

in cells overexpressing the *CKA2* gene, encoding the second catalytic subunit (data not shown). These data are consistent with a role for CKII in the transcriptional regulation of *HSP12* by NC2.

Taken together, the experiments with these mutants demonstrate a difference between NC2-dependent repression in exponential phase and after the diauxic shift, and also between repression and activation by NC2 after the diauxic shift. Furthermore, they suggest a role in vivo for CKII in regulating NC2 function.

Discussion

In this work we analyzed the molecular interactions of the NC2 regulator in cells growing exponentially in glucose and in cells after the diauxic shift. We made the surprising finding that the NC2 α and NC2 β subunits are not associated in a stable complex in exponentially growing cells. This conclusion was reached after purifying the NC2 β subunit to homogeneity, isolating NC2 β complexes of the same size as those identified in total cell extracts, and finding that no NC2 α subunit copurified with NC2 β . Instead, NC2 β forms homo-oligomers, as has previously been observed (Inostroza et al. 1992). Purification of the NC2 α subunit led to similar conclusions, in that no NC2 β subunit could be copurified with

Figure 6. NC2 β is required for repression of *HSP12* in cells growing in glucose and for repression of many genes after the diauxic shift. (A) Wild-type (*NCB2*; MY1) or *ncb2* disrupted cells expressing human NC2 from a centromeric plasmid (*ncb2*; MY1800) were grown to exponential (E) or postdiauxic (PD) phase. Total cellular RNA was extracted and analyzed for the levels of the indicated transcripts. *HIS4* was included as a control in this study because the results have been described (Lemaire et al. 2000). (B). For the experiment presented in Table 2, the association of TBP or NC2 β with the indicated promoters in cells after transfer to medium lacking glucose (PD), relative to in cells growing exponentially (E), was calculated. The results presented are the average of three experiments, and each individual experiment showed the same modulations of promoter occupancy.

the NC2 α subunit from exponentially growing cells. Because both NC2 α and β have been shown to be essential for yeast vegetative growth (Gadbois et al. 1997; Kim et al. 1997; our personal observations), these results suggest that each subunit plays a separate essential role. Alternatively, the subunits do function together at promoters in exponentially growing cells, but without forming a stable complex.

Our findings are surprising, because it has generally been thought that the two subunits of NC2 function together to activate or repress transcription (Goppelt and Meisterernst 1996; Goppelt et al. 1996), to the extent that conclusions about the NC2 regulator have been drawn based upon experiments done with the NC2 α subunit alone (Geisberg et al. 2001) or with the NC2 β subunit alone (Christova and Oelgeschlager 2001). Nevertheless, results that point towards possible distinct or independent roles for the two NC2 subunits can be found in the literature (Inostroza et al. 1992; Yeung et al. 1994, 1997; Mermelstein et al. 1996; Kim et al. 1997, 2000). First, human NC2 β was isolated in the absence of NC2 α and found to form a complex with TBP and DNA as well as to repress transcription in vitro (Inostroza et al. 1992). Secondly, binding of human NC2 β alone to a TBP-DNA complex precluded the association of TFIIB (Yeung et al. 1997). Thirdly, a truncated form of human NC2 β that lacked the histone-fold domain, could repress transcription on its own in vitro (Kim et al. 1997).

Finally, overexpression of NC2 β alone in yeast reduced the total mRNA content (Kim et al. 1997). These experiments point to a possible role for NC2 β without NC2 α . However, there is also evidence for a possible role of NC2 α independently of NC2 β . For example, *sin4* mutation suppresses the lethality of cells lacking NC2 β but not of cells lacking NC2 α (Kim et al. 2000). Interestingly, in plants a role for NC2 α without NC2 β has also been described, in that rice NC2 α can function as a repressor independently of NC2 β (Song et al. 2002).

In contrast to the situation in exponentially growing cells, we find that the α and β subunits of NC2 are associated in a stable complex after the diauxic shift. Interestingly, the conditions that we studied, namely glucose depletion, lead to a large number of changes in cells that are the similar to those induced by a broad range of environmental stress conditions. These stresses in turn lead to specific changes in the expression of specific genes, and these changes can to a large extent be mimicked by the loss of NC2 α function (Geisberg et al. 2001). It will be interesting to determine whether association of the α and β subunits of NC2 is generally regulated by environmental stress conditions. Indeed, if the assembly of a functional NC2 repressor occurs as part of a general response to environmental stress this would nicely explain the relationship between NC2 and regulation of environmental stress genes.

Many phosphorylation events are associated with en-

Table 2. Modulation of the NC2 α /TBP and NC2 β /TBP ratios at promoters

	NC2 α /TBP		NC2 β /TBP	
	E	PD	E	PD
RPS8A	1 $\pm$ 0.4	0.9 $\pm$ 0.07	0.2 $\pm$ 0.07	0.4 $\pm$ 0.15
RPS9	2.4 $\pm$ 0.5	2.6 $\pm$ 0.2	0.1 $\pm$ 0.06	0.4 $\pm$ 0.1
RPS18b	0.9 $\pm$ 0.2	0.9 $\pm$ 0.15	0.2 $\pm$ 0.07	0.4 $\pm$ 0.15
ADH1	1 $\pm$ 0.2	1.3 $\pm$ 0.3	0.2 $\pm$ 0.1	0.3 $\pm$ 0.15
CLN2	1 $\pm$ 0.1	0.8 $\pm$ 0.07	0.5 $\pm$ 0.1	0.6 $\pm$ 0.2
PYK1	2.1 $\pm$ 0.15	2 $\pm$ 0.5	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1
ACT1	2.5 $\pm$ 0.65	2 $\pm$ 0.25	0.3 $\pm$ 0.05	0.4 $\pm$ 0.05
PGK1	1.5 $\pm$ 0.25	1.4 $\pm$ 0.3	0.2 $\pm$ 0.07	0.2 $\pm$ 0.05
RPC40	1.4 $\pm$ 0.1	1.1 $\pm$ 0.02	0.6 $\pm$ 0.25	0.6 $\pm$ 0.2
DBP2	2.2 $\pm$ 0.4	0.6 $\pm$ 0.3	0.3 $\pm$ 0.1	0.6 $\pm$ 0.3
AGA2	1.2 $\pm$ 0.2	0.8 $\pm$ 0.15	0.6 $\pm$ 0.2	0.5 $\pm$ 0.2
CDC31	1.9 $\pm$ 0.45	1.7 $\pm$ 0.4	0.6 $\pm$ 0.25	0.4 $\pm$ 0.15
HSP12	0.9 $\pm$ 0.02	0.8 $\pm$ 0.1	0.6 $\pm$ 0.15	0.2 $\pm$ 0.05
HSP26	1.3 $\pm$ 0.07	1.1 $\pm$ 0.3	0.5 $\pm$ 0.1	0.3 $\pm$ 0.15

Cells expressing tagged NC2 α (MY2493) or tagged NC2 β (MY3352) to functionally complement genomic disruptions of the genes encoding the endogenous subunit were grown to exponential phase in glucose-rich medium (E) and then washed and placed 2 h in rich medium lacking glucose (PD). Cells were then treated with formaldehyde, and TBP (with polyclonal antibodies) or NC2 α or β (with monoclonal antibodies against the HA epitope) were immunoprecipitated from the total cell extract. The amount of the indicated promoter DNA in the immunoprecipitates relative to the total input was calculated. The values obtained for NC2 α or β relative to the values obtained for TBP are indicated. The results presented are the average of three experiments, and each individual experiment showed the same modulations of promoter occupancy.

environmental stress responses, and NC2 is a phosphoprotein whose phosphorylation state has an impact on activity (Inostroza et al. 1992; Goppelt and Meisterernst 1996; Goppelt et al. 1996). Thus, one might expect that protein phosphorylation plays a role in the regulation of NC2 α and β activity. Mutation of putative PKA or PKC sites within NC2 had no observable effect on NC2-dependent transcriptional regulation. In contrast, mutation of putative CKII phosphorylation sites within NC2 α or NC2 β altered certain NC2 functions in vivo. This is consistent with previous experiments in vitro, in which it was shown that phosphorylation by CKII increases the affinity of NC2 for TBP-DNA complexes, over nonspecific DNA binding (Goppelt et al. 1996). The exciting finding is that such mutations reduce repression by NC2 in exponentially growing cells but not NC2-dependent repression after the diauxic shift. This correlates with our observed difference in NC2 complexes before and after the diauxic shift and suggests that phosphorylation by CKII is important to obtain NC2-dependent repression when there are no stable NC2 α / β complexes, possibly because it allows specific binding of NC2 to certain promoters.

We have found a nice correlation between the association of NC2 β and NC2 α in stable complexes, NC2- β -dependent repression of many genes at the diauxic shift, and increased occupancy of repressed promoters by NC2

β relative to NC2 α at this time. We have also found that NC2- β -dependent repression of stress genes in exponentially growing cells correlates with higher occupancy of NC2 β relative to NC2 α on these promoters compared to after the diauxic shift when the stress genes are induced. In fact, in exponentially growing cells, the ratio of NC2 α to NC2 β occupancy is lowest on the repressed *HSP12* promoter compared to all other promoters that we have tested. It seems likely that NC2 β represses the stress genes together with NC2 α , as we have mutants both in the α and in the β subunit that affect *HSP12* repression in exponentially growing cells. This suggests that promoter-bound NC2 α can associate with NC2 β at promoters when repression is required also in exponentially growing cells. However, the nature of the promoter may be very important. For instance, the presence of activator proteins on a given promoter might interfere with the association of NC2 β to promoter-bound NC2 α . In any event, our results show that increased presence of NC2 β relative to NC2 α clearly correlates with repression, not activation.

As others before us, we have found that NC2 α is present at promoters in a way that correlates with the presence of TBP and transcriptional activity (Geisberg et al. 2001). Whether NC2 α associates with TBP-bound promoters in order to be able to associate with NC2 β at the correct time, or whether it plays an active positive role in transcription is not known. The description of a role of NC2 α independently of NC2 β (Kim et al. 2000) would argue against the former possibility. Furthermore, recent experiments in vitro have suggested that the NC2 complex can play a positive role in transcription (Willy et al. 2000). Whether or not NC2 α plays an active positive role at promoters is not clear from our results. In contrast to what others have found (Geisberg et al. 2001) we do not see any difference in the occupancy of promoters by NC2 α relative to TBP at promoters that are up-regulated by NC2 α (such as *DBP2*), down-regulated (*AGA2*, *HSP12*) or unaffected (*PGK1*, *PYK1*, *ACT1* . . .).

Table 3. Modulation of the NC2 β /TBP promoter occupancy measured using polyclonal antibodies

	β /TBP	
	E	PD
HSP12	5.1 $\pm$ 0.3	1.7 $\pm$ 0.07
RPS8A	0.9 $\pm$ 0.2	2.6 $\pm$ 1.1
CLN2	2 $\pm$ 0.15	2.5 $\pm$ 0.75

Wild-type (MY1) cells were grown to exponential phase in glucose-rich medium (E) and then washed and placed 2 h in rich medium lacking glucose (PD). Cells were treated with formaldehyde, and TBP or NC2 β were immunoprecipitated with polyclonal antibodies from the total cell extract. The amount of the indicated promoter DNA in the immunoprecipitates relative to the total input was calculated. The values obtained for NC2 β relative to the values obtained for TBP are indicated in the table. The immunoprecipitation of cross-linked NC2 β with polyclonal antibodies is more efficient (approximately 5–10-fold) than that of epitope-tagged NC2 β with anti-HA antibodies, explaining the higher ratios in this table compared to Table 1.

Figure 7. Analysis of NC2 and CKII mutants for regulation of *HSP12* and *RPS8A* expression. (A) Schematic representation of the NC2 α and β subunits with indication of the amino acid changes created in the different alleles constructed, and indication of the deletion end-point of the *ncb2-2* allele. The mutated sites are putative phosphorylation sites for the kinases indicated above the schematized subunits: casein kinase II (CKII), protein kinase C (PKC), and protein kinase A (PKA). (B,C) The indicated cells were grown to exponential or postdiauxic phases, as indicated, and total cellular RNA was analyzed for the levels of *HSP12*, *DED1* (as an internal control), or *RPS8A* mRNAs. (B) Wild-type cells (WT; MY3223), or cells expressing NC2_{S112A} β (*ncb2-1*; MY3412), NC2_{T64A} α (*bur6-1*; MY3413), or NC2_{C122} β (*ncb2-2*; MY3357) were studied. (C) Wild-type cells (WT; MY1) or cells lacking *CKA1* (*cka1Δ*; YDH6) were studied.

Nevertheless, it is still possible that the presence of NC2 α is important for transcription from certain promoters but not others. This will need to be studied further.

Whether NC2 β has a function *in vivo* independently of NC2 α particularly in exponentially growing cells is unclear. Previous studies have demonstrated that it has the capacity to repress transcription *in vitro* in the absence of NC2 α (Yeung et al. 1997), but whether it actually does so *in vivo* cannot be deduced from our results. Indeed, we have not detected NC2 β in the absence of NC2 α at any promoter analyzed, active or repressed, although we can not rule out the possibility that we have not yet analyzed the right target promoters. Concerning a possible role of NC2 β in activation, a recent study described that human NC2 β could be selectively cross-linked to active but not inactive class II gene promoters in asynchronous cell populations (Christova and Oelgeschlager 2001). However, our studies do not reveal any correlation that might suggest a positive role of NC2 β in transcription in yeast. In one NC2 β mutant, *ncb2-1*, we did observe decreased activation of *HSP12* at the diauxic shift. However, concurrently we also observed a drop in the ratio of NC2 β versus NC2 α at the *HSP12* promoter

at the diauxic shift, which argues against a model in which an NC2 α/β complex contributes to activate the *HSP12* promoter at this time.

Taken together, our observations suggest that a modified view of the NC2 transcriptional regulator is required. According to this model, the NC2 α subunit alone might function on certain promoters as a positive transcription factor independently of the β subunit, and might then be transformed into a factor with repressive functions by association with NC2 β . These studies thus provide an attractive example of how a positive transcription factor might be turned into a negatively acting one by the regulated association with a repressive subunit.

Materials and methods

Strains and media

All strains used are listed in Table 1, and were cultured either in YPD or YP. Strains expressing the NC2 α protein or a truncated NC2 β protein, tagged with a triple HA epitope at the C terminus, were created according to Longtine et al. (1998). The in-

frame fusions, as well as protein expression levels, were controlled by immunoprecipitation with anti-HA antibodies and Western blotting revealed with polyclonal antibodies against NC2 α or β .

To create a plasmid expressing the β subunit of NC2 with an HA epitope at the N terminus, we subcloned the coding sequences of NC2 β in a pBS derivative carrying the HA epitope in the polylinker. The created fusion sequence was subcloned into pPC62, leading it to be expressed under the control of the *ADC1* promoter (pMAC280). A construct expressing the same fusion protein under the control of its own promoter was obtained by amplifying promoter sequences by PCR and subcloning them into pMAC280 between the *ApaI* and *PstI* restriction sites to replace the *ADC1* promoter (pMAC399).

To put a His₁₀-HA epitope tag at the C terminus of NC2 α , a DNA sequence encoding part of the coding region of NC2 α in which the stop codon was replaced by a *SpeI* restriction site was amplified by PCR from the pML12 plasmid (Lemaire et al. 2000), and the PCR product was cloned between the *KpnI* and *SpeI* sites of pMAC303, leading to pMAC312. pMAC312 was digested with *SphI*-*StyI* and cloned into the same sites of pML21 (carrying a genomic clone of the gene) to obtain a *TRP1* centromeric plasmid expressing a complete NC2 α fusion protein from its native promoter (pMAC334).

To obtain a *TRP1* centromeric plasmid expressing a complete NC2 β fusion protein with a His₁₀-HA epitope at the C terminus from its own promoter, the gene was amplified by PCR and cloned between the *KpnI* and *SpeI* sites of pMAC303 (pMAC306). The *KpnI*-*SacI* DNA fragment of pMAC306 was further subcloned into pRS314 (pMAC311). Strains expressing the tagged derivatives in place of the wild-type gene grew like their wild-type counterparts.

Site-directed mutagenesis

We used the QuikChange Directed Mutagenesis kit (Stratagene) and controlled clones by sequencing.

Immunoprecipitation

Whole cell extracts were prepared from 50 O.D.₆₀₀ units of cells growing exponentially (O.D.₆₀₀ < 1.5) or after the diauxic shift (O.D.₆₀₀ 8–10). Cells were broken in lysis buffer (50 mM HEPES at pH 7.5; 300 mM NaCl; 1 mM EDTA at pH 8.0; 0.1% Triton, 1 mM PMSF) with glass beads and vortexing 40 min at 4°C. The lysates obtained were clarified by successive centrifugations. Immunoprecipitations were performed with 1 mg of total protein extract in a final volume of 200 μ L. One microliter of antibodies (anti-HA monoclonal, Babco) and 50 μ L of protein A Sepharose slurry in lysis buffer (1:1) were added before incubating the tubes overnight on a rotator at 4°C. After spinning down the beads and removing the supernatant, the beads were washed 3 $\times$ with an excess of lysis buffer by gently inverting the tubes. The immunoprecipitated proteins were recovered in SDS-PAGE loading buffer and analyzed by Western blot.

Antibodies

Recombinant NC2 α or β were injected into rabbits for antibody production (Elevage scientifique des Dombes).

Chromatin immunoprecipitation

Cells were grown in YPD to an O.D.₆₀₀ between 0.6 and 1.0, extensively washed with water or YP and placed in rich medium with glucose, or without any carbon source, for 1–3 h. Cells

were fixed by addition of formaldehyde to a final concentration of 1% for 30 min at room temperature, followed by addition of glycine to a final concentration of 125 mM. Cells were washed 2 $\times$ in cold TBS, and broken in FA lysis buffer (50 mM HEPES at pH 7.5, 140 mM NaCl, 1 mM EDTA at pH 8.0, 1% Triton, 0.1% sodium deoxycholate) supplemented with 1 mM PMSF as described previously (Strahl-Bolsinger et al. 1997; Kuras and Struhl 1999). The crude extract was sonicated after addition of SDS to a final concentration of 0.5%, cleared, and sonicated again, leading to the Soluble Total Chromatin (STC). An aliquot of the STC (30 μ L) was kept as a control. The immunoprecipitation of DNAs were done on STC diluted 1:5 in FA-lysis buffer. For immunoprecipitation, 1 mL of diluted STC was incubated overnight on a rotator at 4°C with 2 μ L of monoclonal anti-HA antibodies or 0.5 μ L of polyclonal anti-TBP antibodies (purified IgG fraction), or 2 μ L of polyclonal anti-NC2 β antibodies, and 50 μ L of protein A or G Sepharose (a 1:1 slurry in FA lysis buffer). The resin was then sequentially washed with TSE 150 (20 mM Tris at pH 8.0; 2 mM EDTA at pH 8.0; 1% Triton; 0.1% SDS; 150 mM NaCl), TSE 500 (TSE 150, except 500 mM NaCl), buffer III (10 mM Tris at pH 8.0, 1 mM EDTA at pH 8.0, 250 mM LiCl, 1% Igepal, 1% sodium deoxycholate), and, finally, TE. Immunoprecipitated DNA was recovered by incubating twice the resin in elution buffer (1% SDS; 50 mM Tris at pH 7.5; 10 mM EDTA at pH 8.0) at 65°C. The reversal of cross-links, phenol-chloroform extraction and precipitation of the DNA have been described previously (Deluen et al. 2002). Immunoprecipitated DNA was finally resuspended in 25 μ L of TE or H₂O, whereas the aliquot of STC was resuspended in 100 μ L of TE or H₂O. The presence of the *HIS3* and *PHO5* promoters in the immunoprecipitate was determined by radio-labeled PCR, whereas for all other promoters tested, real-time PCR with the SYBR Green dye was used. The sequence of the primers used is available upon request. For the real-time PCR, the DNA recovered in the precipitate was expressed relative to the amount of DNA in the STC (as calculated by dilution curves) in arbitrary units.

Gel filtration

Whole cell extract was prepared in lysis buffer (see above) supplemented with 5% glycerol. Cleared lysate was filtered on 0.45- μ m filter and 300 μ L were loaded on the Superose 6 column (Superose 6 HR10/30, Pharmacia) equilibrated in the same buffer, at a flow rate of 24 mL/h. Four-hundred-microliter fractions were collected. Uneven fractions were precipitated with TCA and pellets were resuspended in 0.1 N NaOH by sonication, mixed with SDS-PAGE loading buffer and analyzed by SDS-PAGE followed by Western blotting. Thyroglobuline (669 kD), apoferritin (443 kD), β -amylase (200 kD), aldolase (158 kD), bovine serum albumin (67 kD), and ovalbumin (44 kD) were used as markers to calibrate the column and eluted at 12.5, 14, 15, 15.75, 16.5, and 17.125 mL, respectively.

NC2 purification

Cultures (inoculated at $\sim 3.10^7$ cells/mL) of 100 L were grown in YPD in fermentators. Half was collected at the exponential phase of growth, whereas the second part was collected after the diauxic shift. Cells were broken in 3 $\times$ lysis buffer [450 mM Tris acetate at pH 7.8; 150 mM potassium acetate; 3 mM EDTA; 60% glycerol; proteases inhibitors (100 $\times$ is leupeptin 0.0284 g/L, pepstatin A 0.137 g/L, PMSF 17 g/L and benzamidin 0.033 g/L)] using a Ball Mill (Dyno-Mill Type KDL-A). Proteins were separated from DNA by ultracentrifugation after potassium acetate precipitation. The protein extract was diluted in the appropriate

buffer before loading onto columns, to reduce the conductivity of the samples.

For strain MY2699, expressing NC2 β -His₁₀-HA, the first step was Bio-Rex 70 (Bio-Rad) anion exchange chromatography. The Bio-Rex 70 was pre-equilibrated in buffer A (10% glycerol, 40 mM HEPES at pH 7.6, 1 mM EDTA, 1 $\times$ protease inhibitors) supplemented with 100 mM KCl. Bound proteins were step-wise eluted with buffer A to which 200, 600, and 1000 mM KCl was added. NC2 β -His₁₀-HA eluted at 600 mM KCl. Those fractions were pooled and submitted to 70% ammonium sulfate precipitation. The pellet was resuspended with 30 mL of buffer A and incubated with 4 mL of protein A Sepharose CL4B-12CA5 monoclonal antibody resin (1–3 mg antibody/mL resin) overnight at 4°C. The resin was collected by flow gravity in a column and washed with 4 mL Ni-buffer (40 mM Hepes at pH 7.6; 10% glycerol; 0.1% Tween 20; 600 mM KOAc; 2 mM β mercaptoethanol and 1 $\times$ protease inhibitors). Bound proteins were eluted 2 $\times$ with 1 mL of Ni-buffer containing 2 mg/mL HA peptide (KKRILYPYDVPDYA) for 15 min at 30°C. The elution fractions were pooled and incubated with 0.4 mL Ni-NTA agarose (QIAGEN) for 4 h at 4°C on a rotating wheel. The resin was collected by gravity flow in a disposable column and washed with 2 mL of Ni-buffer. Bound proteins were eluted successively with 10 $\times$ 75 μ L fractions of Ni40-buffer (40 mM imidazole; 40 mM Hepes at pH 7.6; 10% glycerol; 0.1% Tween 20; 200 mM KOAc; 2 mM β mercaptoethanol; 1 $\times$ protease inhibitors) and Ni500-buffer (same except 500 mM imidazole, 300 mM KOAc).

For strain MY2407, expressing NC2 β with a single HA epitope at the N terminus from a heterologous promoter, the flow-through of the Bio-Rex 70 was loaded onto DEAE Sepharose, pre-equilibrated in buffer B (10% glycerol 20 mM Tris acetate at pH 7.8, 1 mM EDTA, 1 $\times$ protease inhibitors) supplemented with 100 mM KCl. Bound proteins were step-wise eluted with buffer B supplemented with 200 and 600 mM KCl. NC2 β complexes eluted in 200 mM KCl. Those fractions were pooled and precipitated as before, and then subjected to 12CA5-affinity purification as described above. The eluate from the 12CA5-affinity resin was further purified on nickel-agarose. This step was done by mistake as NC2 β in this strain was not tagged with multiple histidines, and although NC2 β did bind the resin, the nickel-agarose step did not contribute to any further enrichment. The fractions eluted from nickel-agarose were aliquoted and stored at -80°C .

For strain MY2952, expressing NC2 α -His₁₀-HA, the protein extracts were diluted with buffer B, and loaded first to the Bio-Rex 70 resin pre-equilibrated in buffer A containing 100 mM KCl. The flow through of the Bio-Rex 70 went directly on top of the DEAE resin pre-equilibrated in buffer B containing 100 mM KCl. Proteins bound to the DEAE resin were step-wise eluted with buffer B containing 200 mM and 600 mM KCl. NC2 α -containing complexes were found essentially in the 200 mM KCl fractions of the DEAE. Those fractions were pooled and precipitated as mentioned above. The precipitated material was then subjected to HA-epitope-affinity followed by nickel-affinity chromatographies as described previously.

S1 analysis of total cellular RNA

Total RNA was prepared and analyzed as described previously (Collart and Struhl 1993). Oligonucleotide sequences are available upon request.

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Sandrine Creton, Jesper Q. Svejstrup and Martine A. Collart

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