A single point mutation in TFIIA suppresses NC2 requirement *in vivo*

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Negative cofactor 2 (NC2) is a dimeric histone-fold complex that represses RNA polymerase II transcription through binding to TATA-box-binding protein (TBP) and inhibition of the general transcription factors TFIIA and TFIIB. Here we study molecular mechanisms of repression by human NC2 *in vivo* in yeast. Yeast NC2 genes are essential and can be exchanged with human NC2. The physiologically relevant regions of NC2 have been determined and shown to match the histone-fold dimerization motif. A suppressor screen based upon limiting concentrations of NC2 β yielded a cold-sensitive mutant in the yeast TFIIA subunit Toa1. The single point mutation in Toa1 alleviates the requirement for both subunits of NC2. Biochemical characterization indicated that mutant (mt)-Toa1 dimerizes well with Toa2; it supports specific recognition of the TATA box by TBP but forms less stable TBP–TFIIA–DNA complexes. Wild-type but not the mt-Toa1 can relieve NC2 effects in purified transcription systems. These data provide evidence for a dimeric NC2 complex that is in an equilibrium with TFIIA after the initial binding of TBP to promoter TATA boxes.

Keywords: negative cofactor 2/suppressor screen/transcriptional activation/transcriptional repression

Introduction

Regulation of RNA synthesis uses inhibitory and stimulatory pathways both in prokaryotes and eukaryotes. In prokaryotes, repression is probably the major control mechanism that is usually realized through direct inhibition of RNA polymerases during initiation or elongation (Mishra and Chatterji, 1993; Eick *et al.*, 1994). The situation in eukaryotes is more complex. This is mainly a consequence of the demands on the packaging of the genome and the gene regulatory program during differenti-

ation of multicellular organisms. Chromatin is thought to impose a mostly inhibitory barrier that can be controlled by several enzymatic activities, some of which maintain or strengthen the inhibitory impact on transcription. Recent examples include methyltransferases (Kass and Wolffe, 1998; Wolffe, 1998) and histone deacetylases (Kuo and Allis, 1998; Wade *et al.*, 1998; Zhang *et al.*, 1998). Secondly, gene activation in vertebrates usually requires several regulatory factors that function in a synergistic manner. This generates additional possibilities for negative control of transcription. DNA-binding inhibitors may interfere with the formation of regulatory complexes by inhibiting the binding of essential activators to genes. Alternatively repressors may preclude the interaction of activating complexes with the basal transcription machinery (Hanna and Hansen, 1996).

One intriguing difference to prokaryotes is the very large size of the transcription machinery and the existence of numerous accessory factors in eukaryotes. Many of the site-specific DNA-binding regulatory factors function through accessory factors. These can be tightly associated with the transcription machinery or reside in independent entities. Cofactors are required in all the steps during gene activation and silencing. They include modifying enzymes, bridging factors and other functional components that help to establish or interfere with the formation of active transcription complexes (Kaiser and Meisterernst, 1996; Stargell and Struhl, 1996; Lee *et al.*, 1998; Hampsey and Reinberg, 1999). One main target of cofactors is the basal transcription apparatus. A well studied example is the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II, which is subject to regulation by both protein kinases (Payne and Dahmus, 1993; Marshall *et al.*, 1996; Lee and Greenleaf, 1997) and phosphatases (O'Brien *et al.*, 1994; Archambault *et al.*, 1997; Cho *et al.*, 1999). RNA polymerase II is inhibited through phosphorylation before initiation, whereas elongation of many if not all genes is thought to require phosphorylation through kinases (Hengartner *et al.*, 1998; Lee and Lis, 1998).

Another group of repressors targets the machinery through direct interaction without binding itself to DNA. The first example reported for such a repressor in eukaryotes is the negative cofactor 2 (NC2), which is also known as the Dr1–DRAP complex (Meisterernst and Roeder, 1991; Inostroza *et al.*, 1992). NC2 was identified in extracts of human tumour cells as an activity that inhibits RNA polymerase II through binding to the TATA-box-binding protein (TBP). One model implies that NC2 forms an alternative partial preinitiation complex consisting of TBP, NC2 and the promoter. NC2 competes with TFIIA and subsequently prevents the assembly of TFIIB and the other general transcription factors. This leads to termination of initiation of transcription (Goppelt *et al.*,

1996). NC2 was also shown to inhibit RNA polymerase III but not RNA polymerase I transcription (White *et al.*, 1994). The underlying molecular mechanisms remain unknown.

The NC2 complex consists of two subunits containing histone-fold motifs that are closely related to the histones H₂A and H₂B core regions. The histone-fold region is also closely related to the CBF–NF-Y and the yeast HAP complex, respectively (Goppelt *et al.*, 1996; Kim *et al.*, 1996; Sinha *et al.*, 1996). It has been proposed that NC2 dimerizes through this histone-fold motif, adopting a structure related to the histones in the nucleosome (Arents and Moudrianakis, 1995; Tan *et al.*, 1996). *In vitro* and *in vivo* dissection of NC2 revealed the importance of the histone-fold regions for repression and for binding to TBP (Inostroza *et al.*, 1992; Yeung *et al.*, 1994, 1997; Goppelt *et al.*, 1996; Kim *et al.*, 1997). C-terminal regions of the NC2 β subunit (Dr1) are important for stability of the complex and for interaction with TBP (Yeung *et al.*, 1994, 1997) and DNA (Goppelt *et al.*, 1996).

NC2 seems to be restricted to the eukaryotic kingdom where it is highly conserved through evolution. Homologues are known in yeast and the nematode *Caenorhabditis elegans*. In yeast, NC2 was isolated both genetically and biochemically (Goppelt and Meisterernst, 1996; Gadbois *et al.*, 1997; Yeung *et al.*, 1997; Lee *et al.*, 1998). The human NC2 β (Dr1) but not NC2 α (DRAP) complemented its yeast counterpart (Kim *et al.*, 1997). Interestingly, mutations in NC2 α suppress mutations in components of the RNA polymerase II holoenzyme, arguing for an equilibrium between positive mediator proteins and a negative repressor *in vivo* (Gadbois *et al.*, 1997). However, our current understanding of NC2 function is mostly derived from *in vitro* studies whose physiological relevance remains to be demonstrated.

Towards this goal we have now studied human NC2 in yeast. We show that both NC2 genes are required for yeast viability. Either one or both yeast subunits as a pair can be exchanged against their human counterparts. The essential regions of human NC2 in yeast were defined. The conserved histone fold and part of the C-terminal regions in NC2 β are necessary for yeast growth. A suppressor screen was initiated by offering yeast low concentrations of human NC2 β resulting in a slow growth phenotype. The latter was spontaneously suppressed by mutation in the large yeast TFIIA subunit, Toa1 (Ranish and Hahn, 1991; Kang *et al.*, 1995). In the suppressor strain yeast loses the requirement for both subunits of NC2, whereas TFIIA remains essential. The suppressor carrying a single point mutation in the dimerization surface with its second subunit, Toa2, was characterized by biochemical means. The suppressor is deficient in formation of stable TBP–TFIIA-promoter complexes. These data provide *in vivo* evidence for a dimeric NC2 complex that functions to counteract stimulatory factors under standard growth conditions. They further support the idea of a dual function of TFIIA and provide indirect evidence for an equilibrium between a dimeric NC2 complex and TFIIA in living cells.

Results

Functional analysis of human NC2 in yeast

To study the molecular mechanism underlying repression of transcription by the dimeric human NC2 α –NC2 β

(DRAP–Dr1) complex *in vivo*, we utilized the yeast *Saccharomyces cerevisiae*, which contains two highly related genes (Goppelt *et al.*, 1996; Gadbois *et al.*, 1997; Kim *et al.*, 1997; Prelich, 1997). Previous experiments have demonstrated that both subunits of the global NC2 repressor are essential for *S. cerevisiae* vegetative growth (Kim *et al.*, 1997; Prelich, 1997). The human NC2 β (hNC2 β) subunit expressed in yeast complemented a disruption of the essential yeast NC2 β (yNC2 β) gene, although not very efficiently (Kim *et al.*, 1997). Surprisingly, hNC2 α did not complement yNC2 α , although hNC2 α was active in combination with yNC2 β *in vitro* (Goppelt and Meisterernst, 1996). Here we used a plasmid shuffle strain for NC2 α (MY1784, see Table I) or NC2 β (MY1780, see Table I) in which the genomic copy was disrupted and which carried instead an episomal copy of the individual yNC2 gene on a *URA3* centromeric plasmid (M.Lemaire, J.Xie, M.Meisterernst and M.Collart, in preparation). Plasmid shuffle strains were transformed with *LEU2* centromeric plasmid carrying the hNC2 α or with *TRP1* high-copy plasmid carrying the hNC2 β gene, respectively (pJX1 and pJX8, see Table II). The transformants were streaked on minimal media containing 5-fluoroorotic acid (FOA) to select for cells that lack the yNC2 genes. Healthy yeast cells were obtained when the null allele of yNC2 β was transformed with a high-copy plasmid expressing hNC2 β or when the null allele of yNC2 α was complemented by a low-copy plasmid expressing hNC2 α (pJX1, Figure 1A). Thus, in agreement with previous findings (Prelich, 1997) both NC2 genes are essential for normal yeast growth. However, in contrast to a former report (Kim *et al.*, 1997) hNC2 α also complemented its yeast counterpart.

A third strain was generated that carried a disruption of both genomic yNC2 genes and a single *URA3* centromeric plasmid expressing both the yeast genes (MY1791, Table I). The expression of both human subunits from pJX1 and pJX8, but not the expression of either human subunit alone, efficiently complemented the disruption of yNC2 genes. The double knockout strain for yNC2 α and yNC2 β genes (MY1791) was subsequently used for a structure–function analysis of the human NC2 complex in yeast. While this approach may not unravel all possible pathways through which NC2 might operate in mammals, it was expected to provide insight into the evolutionarily conserved basal mechanism of repression. Various deletion mutants, summarized in Figure 1B, were generated, cloned into a yeast expression vector (see Materials and methods; Table II) and shuffled into the double knockout strain. In brief, these experiments show that at least both histone-fold domains, as well as a short region C-terminal of the histone-fold domain of hNC2 β , are required for yeast growth. These regions match the conserved regions between human and yeast (Goppelt and Meisterernst, 1996). Notably, part of the repression region of human NC2 β (amino acids 130–176) and the entire C-terminal region of human NC2 α (amino acids 81–205) are not required for normal yeast growth. This is consistent with results obtained with the yNC2 genes (Prelich, 1997).

Isolation of a suppressor of low levels of NC2 β

Yeast displayed a slow growth phenotype if hNC2 β was supplied on a low-copy plasmid. This phenotype was used

Table I. Yeast strains

Strains	Genotype	Source
MY1 (KY803)	<i>MATa trpΔ1 ura3-52 gcn4Δ leu2::PET56 gal2</i>	Hope and Struhl (1986)
MY2 (KY804)	isogenic to MY1 except <i>MATα</i>	Collart and Struhl (1994)
MY4	isogenic to MY1 except <i>MATα his3::TRP1</i>	this study
MY1780	isogenic to MY1 except <i>ync2β::KAN + pURA3-yNC2β</i>	this study
MY1781	isogenic to MY1780 except <i>MATα</i>	this study
MY1784	isogenic to MY2 except <i>ync2α::KAN + pURA3-yNC2α</i>	this study
MY1791	isogenic to MY2 except <i>his3::TRP1 ync2α::KAN ync2β::KAN + pURA3-yNC2α-yNC2β</i>	this study
JY20	isogenic to MY1 except <i>ync2β::KAN + pMAC102-hNC2β</i>	this study
MY1802	isogenic to JY20 except <i>toa1-2</i>	this study
MY1855	isogenic to JY20 except <i>MATα his3::TRP1</i>	this study
MY1870	isogenic to MY1 except <i>toa1-2</i>	this study
MLY542	<i>a/α</i> diploid isogenic to MY1 except <i>his3::TRP1/HIS3</i>	
MLY31	isogenic to MY542 except <i>ync2α::KAN /yNC2α</i>	this study
MLY462	<i>a/α toa1::URA3/TOA1 HIS3/his3::TRP1</i>	this study
MLY465	<i>MATa toa1::URA3 his3::TRP1 + pML83</i>	this study
MLY504	<i>MATa toa1::URA3 his3::TRP1 ync2β::KAN + pML83</i>	this study

Table II. Plasmids

Plasmid	Description
pMAC102	shuttle vector with <i>ADHI</i> promoter and 3'-untranslated sequences <i>CEN LEU2 Amp</i>
pJX1	<i>hNC2α</i> full length in pMAC102
pJX3	<i>hNC2β</i> full length in pMAC102
pJX4	<i>hnc2α</i> (amino acids 8–88) in pMAC102
pJX5	<i>hnc2α</i> (amino acids 1–77) in pMAC102
pJX6	<i>hnc2α</i> (amino acids 8–125) in pMAC102
pJX23	<i>hnc2α</i> (amino acids 8–80) in pMAC102
pJX14	shuttle vector with <i>ADHI</i> promoter and 3'-untranslated sequences 2μ <i>TRP1 Amp</i>
pJX8	<i>hNC2β</i> full length in pJX14
pJX9	<i>hnc2β</i> (amino acids 1–83) in pJX14
pJX20	<i>hnc2β</i> (amino acids 1–112) in pJX14
pJX26	<i>hnc2β</i> (amino acids 10–129) in pJX14
pML83	<i>toa1(V251F)</i> from MY1802 in pBS SK(+)
pJX27	<i>toa1(V251F)</i> in pET15b

to select for spontaneous suppressors of the lowered NC2 level. Fast growing colonies were analysed by genetic standard procedures for the presence of single suppressor mutations, as explained in detail in the Materials and methods section. One of the strains (MY1802) displayed a slightly cold-sensitive phenotype at 16°C but grew similarly to wild-type yeast strain at 30 and 37°C (Figure 2A). Crossing of MY1802 with the parental wild-type strain MY4 and subsequent tetrad analysis demonstrated that the cold-sensitive phenotype was retained both in the absence and presence of the *yNC2β* gene. Thus, NC2β is not necessary for viability in the presence of the suppressor. Notably, the suppressor also alleviates a requirement for NC2α. This was shown through tetrad analysis of sporulated diploids of the suppressor MY1870 crossed with MY1784, which lacks a genomic copy and carries an episomal copy of the *yNC2α* gene (see Materials and methods for experimental details). Taken together, the suppressor mutation generates a yeast strain that does not require the NC2 complex for cell viability.

The suppressor strain carries a point mutation in *Toa1*

During tetrad analysis of the suppressor MY1802 crossed with its parental wild-type strain we noted tight co-

segregation of the suppressor mutation with the *HIS3* locus. The suppressor mutation was obtained in a strain that carries a wild-type *HIS3* locus (JY20, see Table I). When JY20 was crossed to MY4, which carries a mutated *his3* locus (*his3::TRP1*), out of 32 tetrads all progeny carrying the suppressor mutation were His⁺, with only three tetrads that had the genotype *his3::TRP1*. This result could be explained if the suppressor is a gene located in the proximity of the *HIS3* locus. The *Toa1* gene encoding the large subunit of yeast TFI_{II}A, is located at a distance of ~20 kb from *HIS3* on chromosome XV. It appeared to be a promising candidate gene, given that TFI_{II}A had been shown to compete with NC2 for binding to TBP *in vitro* (Goppelt *et al.*, 1996). To test this assumption, the *Toa1* gene was amplified from the suppressor strain by PCR. Three independent clones were isolated, sequenced and shown to carry a single mutation at amino acid position 251. Amino acid 251 is valine in wild-type (wt)-*Toa1*, whereas the mutant contained a hydrophobic exchange valine to phenylalanine at position 251 (Val251Phe) (the technical term is *toa1-2*, see Table I, but for simplicity the suppressor is subsequently called mt-*Toa1* and mt-*Toa1* gene, respectively). To confirm that the mutation is sufficient to bypass the essential need for NC2, we created a diploid strain heterozygously disrupted for the NC2β gene and the *Toa1* gene and carrying an episomal copy

Fig. 1. Complementation of yeast NC2 with its human counterparts. Effects of hNC2 wild-type and deletion mutants on yeast growth. Dilutions of equal numbers of cells (as measured at OD₆₀₀) of each strain were spotted on selective media with or without FOA. (A) Both NC2 genes are essential for cell viability and expression of hNC2 α in a genomic deletion of yNC2 α (indicated as yNC2 α URA) or hNC2 β in a genomic deletion of yNC2 β (indicated as yNC2 β URA) restores viability of yeast. The control is the wt strain, MY1. (B) Deletion analysis of hNC2 α and hNC2 β in a double knockout strain. Mutants in one subunit were analysed together with the respective wt partner. '+' and '-' indicate the consequences on cell growth. Characteristics of regions in both subunits are indicated schematically. hNC2 α deletion mutants were expressed in yeast on a low-copy vector and hNC2 β on a high-copy vector.

of the mt-Toa1 gene (by crossing MLY465 with MY1781). This strain was sporulated and tetrads dissected. Spores carrying the NC2 β gene and the Toa1 gene disruptions and the plasmid expressing the mt-Toa1 gene were obtained (MLY504, Table I, see Materials and methods for details). Thus, the mutation identified in the Toa1 gene is capable of suppressing the need for NC2 β .

Toa1 suppressor mutant dimerizes with Toa2

In the X-ray crystal structure of yeast TFIIA, TBP and DNA (Geiger *et al.*, 1996; Tan *et al.*, 1996), Val251 is positioned on the dimerization surface of the yTFIIA complex (Figure 2B). To test whether mt-Toa1 is deficient in dimerization, wt- and mt-Toa1 subunits were expressed in *Escherichia coli* and complex formation was analysed. Insoluble overexpressed proteins were purified and co-renatured as described in Materials and methods and fractionated on sizing columns. Wild-type and mt-Toa1 co-eluted precisely with the small Toa2 subunit on a

SMART Superdex 75 column (Figure 3A). Free Toa2 and Toa1 that presumably remain insoluble in the absence of the corresponding partner were not detected. Native wt- and mt-yTFIIA complexes migrated indistinguishably from *E. coli*-expressed proteins and from each other on the gel filtration column, as demonstrated through fractionation of total yeast extracts and monitoring of the small subunit Toa2 (Figure 3B). The mt-Toa1 also behaved indistinguishably from wild type when we analysed protein interactions with proteins expressed in reticulocyte lysates as well as in mammalian two-hybrid assays (data not shown). Thus, despite the position of Val251 on the dimerization surface, mt-Toa1 is competent in dimerization with its partner Toa2. One possible explanation is that the large dimerization surface within the yTFIIA complex tolerates mutations (Tan *et al.*, 1996; see Figure 2B).

mt-Toa1 displays defects in TBP-DNA complex formation

Val251 is close to both the DNA-binding- and the TBP interaction surface of yTFIIA. Thus, exchange of valine with phenylalanine might affect ternary complex formation. To test this hypothesis we analysed yTFIIA complexes in gel shift assays together with recombinant human TBP. Wild-type and mt-yTFIIA complexes were titrated in the presence of constant amounts of human TBP and a TATA box-containing promoter fragment. Compared with wt, reduced complex levels were seen with mutant in a large concentration range of yTFIIA (Figure 4A, compare lanes 3–8 with lanes 9–14). This was independent of the concentration of both TBP and DNA (Figure 4B, compare lanes 2–4 with lanes 5–7; data not shown). Steady-state levels of TBP-yTFIIA-DNA complexes at saturating concentrations of yTFIIA were clearly reduced in the mutant. Yeast TBP behaved similarly to hTBP but not identically. yTBP binds itself to DNA in the absence of TFIIA complexes (Figure 4B, lane 14). yTFIIA forms a complex with yTBP that has lower mobility. This complex is not detected in the presence of mt-Toa1 (Figure 4B, lanes 8–10 versus lanes 11–13). We have no indications for differences in direct TFIIA-DNA contacts. Furthermore, differences in direct interactions with TBP were not seen when wt- and mt-yTFIIA complexes were passed over immobilized GST-TBP (data not shown). In summary, the single point mutation Val251Phe in Toa1 generates a complex that is specifically deficient in ternary complex formation.

The suppressor supports binding of TBP to the TATA box

To analyse further the molecular structure of the ternary TBP-yTFIIA-DNA complex we performed DNase I-footprinting experiments. Under conditions where yeast TBP shifts a promoter oligonucleotide the TATA box was not protected against digestion by the enzyme (Figure 5A, lane 2 versus lanes 3 and 4). Instead, non-specific protection of DNA was seen (Figure 5A, compare lanes 2 and 3 in the lower part). Addition of the yTFIIA complex induced full protection of the region surrounding the TATA box (Figure 5A, lanes 4 and 5) depending on TBP (compare lanes 3, 8 and 9 with lanes 4 and 5). Surprisingly, comparable effects were seen with the mt-yTFIIA complex at identical concentrations (Figure 5A, compare lanes 4

Fig. 2. A cold-sensitive suppressor mutation in *Toa1*. (A) The suppressor (MY1802) of low concentrations of NC2 β , carrying a single point mutation, shows a cold-sensitive phenotype at 16°C whereas it grows similarly to control strain (MY1) at 30 and 37°C on YPD medium. (B) Position of the suppressor mutation in the yTFIIA-TBP-DNA complex. The X-ray crystal structure comprises part of the large yTFIIA subunit *Toa1* (cyan) and the small subunit *Toa2* (yellow) complexed with TBP (blue) bound to promoter DNA (white). Mutated Val251 (indicated as V251) on the *Toa1*-*Toa2* dimerization surface is depicted in red.

and 5 with lanes 6 and 7). Thus, both wt- and mt-*Toa1* facilitate binding of yeast TBP to the TATA box. Similarly, wt- and mt-yTFIIA complexes performed essentially identically on the coding strand (Figure 5C). Given the differences in gel shift experiments yeast and human TBP were compared in footprinting experiments (Figure 5B). Intact and mt-yTFIIA complexes facilitated quantitative binding of human TBP and yeast TBP (Figure 5B, lanes 4 and 5 versus lanes 7 and 8). Furthermore, similar patterns were observed when we analysed DNA binding of wt- and mt complexes with the phenanthroline-copper technique in gels, arguing against major structural differences between wt- and mt complexes (data not shown). This is in contrast to the gel shift results where mt-yTFIIA does not bind yTBP (Figure 4B). Moreover, the number of mt-yTFIIA-TBP-DNA complexes in gel shifts is small, or absent in

the case of yTBP, whereas the majority of fragments is protected in the footprint under identical conditions. Taking into account that protein-DNA complexes must withstand various forces during electrophoresis, we suggest that the mutation in *Toa1* renders the ternary complex less stable. Nevertheless, the suppressor maintains the ability to support the initial binding of TBP to the promoter.

***Toa* mutant is deficient in anti-repression of NC2 inhibition of transcription**

One important open question concerns the impact of the mutation of *Toa1* on transcription. TBP and TFIIA have previously been shown to be exchangeable between yeast and man (Hahn *et al.*, 1989). Having a reconstituted mammalian transcription system to hand, and lacking a comparable yeast system, we tested the yTFIIA complexes

Fig. 3. The suppressor mutation in Toa1 does not prevent dimerization of yTFIIA. (A) Silver-stained SDS-gel showing co-elution of *E. coli* expressed and co-renatured wt- and mt-yTFIIA (Toa1 and Toa2) complexes on Superdex 75. Lane M contains marker proteins of the indicated sizes. (B) Western blot analysis of fractionated yeast extracts. The small yTFIIA subunit Toa2 is monitored with anti-Toa2 antibodies to demonstrate co-elution of TFIIA in fractionated yeast extracts from wt-yTFIIA (MY1-) and mt-yTFIIA (MY1870)-expressing strains on Superdex 75. Antibodies against the large TFIIA subunit were not available. Note, however, that native wt- and mt-yTFIIA complexes from yeast extracts elute indistinguishably from the *E. coli* expressed complexes analysed in (A) in between the 66 and 21.5 kDa markers. Molecular weight markers were fractionated under identical conditions.

in a human system. NC2 effectively represses transcription in crude nuclear extracts that contain a more physiological mixture of general transcription factors, including the TFIID complex and accessory factors. In fact NC2 is even more active in the crude systems than in a purified system that contains TFIID instead of TBP (data not shown; Goppelt *et al.*, 1996). However, crude systems contain human TFIIA and an undefined mixture of TBP-containing and TBP-related complexes (Hansen *et al.*, 1997; Brand *et al.*, 1999). Hence, we used a more purified system deficient of TFIIA together with recombinant TBP, NC2 and TFIIA. NC2 is a potent repressor of basal transcription in this system, both on an HIV core promoter and on an adenovirus major late promoter (ML) (Figure 6, lane 1 versus lane 2). Addition of yTFIIA to the reactions alleviated the repression of NC2 on the ML (Figure 6, compare lane 4 with the control in lane 8) and reduced it on the HIV core promoter (lanes 3 and 4). In contrast, mt-yTFIIA was completely inactive in relieving inhibition by NC2 (Figure 6, lanes 5 and 6). Experiments conducted with yeast instead of human TBP yielded identical results (data not shown). Taken together, TFIIA has been modified in the suppressor strain in a way that eliminates the NC2 anti-repression pathway and reduces its potential to form stable complexes with TBP on DNA, but maintains its capacity to support binding of TBP to the TATA box.

Fig. 4. Influence of the mutation in Toa1 on binding of yTFIIA to TBP on DNA. Gel shift analysis with recombinant purified TBP, wt-yTFIIA and mt-yTFIIA. Components and the position of complexes and free DNA are indicated. (A) Titration of wt- and mt-yTFIIA (1, 2.5, 10, 25, 37.5 and 50 ng) in the presence of 30 ng of hTBP. (B) Comparison of hTBP (lanes 2-7) and yTBP (lanes 8-14). In titrations 5, 10 and 20 ng of hTBP or yTBP were added to 2.5 ng of recombinant wt- or mt-yTFIIA. Note that the yTFIIA-yTBP complex is absent if mt-Toa1 is included. Higher order complexes (h.o.) may result from binding of a second yTFIIA-TBP complex to DNA.

Discussion

General factors and basal cofactors have mostly been discovered through biochemical *in vitro* experiments followed by reverse genetics. The general character in many cases has hampered their characterization *in vivo*. On the other hand, many of the mutations that were isolated in genetic screens proved to encode basal transcription factors and components of accessory complexes of RNA polymerase II, usually evident after their isolation and biochemical characterization (Grant *et al.*, 1997, 1998; Roberts and Winston, 1997; Orphanides *et al.*, 1999; Sterner *et al.*, 1999). The NC2 (Dr1-DRAP) complex went through a similar process. NC2 has initially been identified in extracts of mammalian cells where it functioned as a repressor of basal transcription. Biochemical experiments led to a model for the molecular mechanism by which NC2 represses transcription (Meisterernst and Roeder, 1991; Inostroza *et al.*, 1992) and to the cloning of the complex (Inostroza *et al.*, 1992; Kim *et al.*, 1997). In parallel, NC2 α was discovered as a suppressor of mutations in SRB4 (Goppelt and Meisterernst, 1996; Gadbois *et al.*,

Fig. 5. Analysis of the suppressor in DNase I footprinting. Minimal protected regions, as assigned by an A+G sequence reaction, are marked by boxes. Non-transcribed strand of a 60 bp HIV core promoter (A and B) and transcribed strand of a 90 bp HIV core promoter fragment (C) were used. Reactions contained 50 ng of human- or yeast TBP (hTBP, yTBP) if indicated. (A) Titration of yTFIIA: 10 and 25 ng of wt-yTFIIA (wt) and mt-yTFIIA (mt) were included in the reactions in the presence and absence of yTBP as indicated. (B) Comparison of yeast and human TBP. Reactions contained 25 ng of wt- and mt-yTFIIA (IIA), respectively. Lanes 9–10 show control reactions in the absence of TBP. (C) Footprinting analysis of yTFIIA–hTBP complexes on the transcribed strand of the HIV promoter. Reactions contained 10 ng of wt- and mt-yTFIIA (IIA), respectively.

Fig. 6. Wild-type yTFIIA (lanes 3 and 4) but not mt-yTFIIA (lanes 5 and 6) relieves the transcriptional inhibition of human NC2 *in vitro*. HIV and ML core promoters were transcribed in a partially purified transcription system as detailed in Materials and methods. NC2 (70 ng), wt-yTFIIA (2.5 ng) and mt-yTFIIA (7.5 ng) were added where indicated.

1997; Prelich, 1997; Lee and Lis, 1998). The fact that SRB4 functions as a general coactivator (Holstege *et al.*, 1998) suggested a global negative role of NC2 in pol II transcription and introduced NC2 as a putative antagonist of the RNA polymerase II holoenzyme (Gadbois *et al.*, 1997). However, it seemed difficult to rule out that inactivation of NC2 merely compensates for the reduced activity of a positive mediator. Indeed, NC2 might well function through specific genes and it could antagonize other components in the presence of intact SRB4.

Mutations in TFIIA suppress the requirement for NC2 *in vivo*

In this work we have isolated yeast TFIIA subunit Toa1 as a suppressor of NC2 function *in vivo*. The suppressor fully overcomes the need for NC2 in yeast. Yeast loses

the requirement for NC2 in the presence of the suppressor, but it further depends on TFIIA. Thus, TFIIA must have functions other than competing with NC2. A general role of TFIIA on many if not all yeast genes has been predicted previously (Ranish and Hahn, 1991; Kang *et al.*, 1995; Ozer *et al.*, 1998). Moreover, TFIIA has been thought to play a role in activation of transcription both in yeast and in mammals (Hahn *et al.*, 1989; DeJong and Roeder, 1993; Stargell and Struhl, 1996; Olave *et al.*, 1998). The mt-Toa1 may help in the future to dissect the different functions of TFIIA.

A mechanism for repression by NC2 in yeast

The finding that mutations in one TFIIA subunit suppress the need for NC2 is a strong argument that the two factors affect the same process in living cells. Based upon the previous molecular characterization (Meisterernst and Roeder, 1991; Goppelt *et al.*, 1996), one might predict that the mutation should in some way affect the equilibrium between TFIIA, NC2 and TBP. Proof for this model can only come from *in vitro* experiments. Towards this goal we have studied the suppressor mutant with recombinant proteins in biochemical assays. We showed that a single point mutation in Toa1 has reduced capacity to form stable complexes with TBP and DNA in gel shift experiments. On the other hand, the mutant could efficiently recruit TBP to the promoter TATA box. Most of the TBP–DNA complexes seen in footprints were unstable in gel shift experiments. Our data further suggest that TBP initially binds randomly to the DNA. Binding to the TATA box is much more efficient in the presence of TFIIA. The mutation in Toa1 does not significantly interfere with this function of TFIIA. However, complexes that contain the single point mutation in Toa1 appear to be less stable.

It has been suggested that TBP undergoes conformational changes on promoter DNA (Chi and Carey, 1996; Coleman and Pugh, 1997; Jackson *et al.*, 1999). In light of these findings one might discuss whether the stable complexes seen in gel shifts represent a small isomerized subpopulation that escapes detection in footprinting experiments. Indications for such a subpopulation could be specific DNase I-hypersensitive sites or footprinting differences in the shifted population. If one extends this picture one might further hypothesize that TFIIA not only helps to recruit TBP but subsequently induces or stabilizes isomerization of the complex. However, with the present set of experiments we could not obtain novel conclusive evidence for such a scenario. Indeed, the DNA contacts of wt- and mt-Toa1-containing complexes are very similar. Stability defects could result from a lever effect mediated by the change in the side chain (Val251Phe) leading to small local variations at the DNA- and TBP-binding surface (Geiger *et al.*, 1996; Tan *et al.*, 1996). Alternatively or in addition, proper folding of the central part of Toa1 (which is missing in the X-ray structure of Richmond and colleagues; Tan *et al.*, 1996) could be affected.

Structural and functional relationship of human and yeast proteins

The close relationship between human and yeast NC2 proteins can be used to study molecular pathways *in vivo*. Both human NC2 subunits function to complement the essential role of the corresponding yeast genes. These observations extend earlier reports demonstrating that human Dr1 complements the yeast gene (Kim *et al.*, 1997). Remarkably, even the heterologous combinations (human NC2 α and yeast NC2 β and vice versa) generate viable yeast cells. Thus, if NC2 indeed functions as a dimer, human and yeast proteins must heterodimerize through the histone-fold domains *in vivo*. Our analysis of a suppressor in yeast TFIIA subunit Toa1, which was isolated based upon limiting concentration of one NC2 subunit but that alleviated the need for both NC2 subunits, lends additional support to this hypothesis. We have also generated an altered specificity mutant that provides direct evidence for a requirement for both subunits for growth of yeast (J.Xie and M.Meisterernst, unpublished data).

Structure–function analysis not unexpectedly demonstrated a need for the regions that are conserved between human and yeast proteins (Goppelt and Meisterernst, 1996). These are the histone-fold domain (Xie *et al.*, 1996; Luger *et al.*, 1997; Birck *et al.*, 1998; Lavigne *et al.*, 1999) in the α subunit and the histone fold together with a short 50 amino acid C-terminal region in the β subunit. The modulatory role of the C-terminal regions of both subunits in mammalian systems *in vitro* may thus be restricted to higher eukaryotes (Yeung *et al.*, 1994, 1997; Goppelt *et al.*, 1996; Ikeda *et al.*, 1998). These data also show that the basal functions are entirely mediated through the core of NC2 and they are preserved through evolution.

Transcriptional analysis of the suppressor mutant

The mt-Toa1 is inactive in transcriptional anti-repression, whereas wt-Toa1 fully relieves NC2 effects. This unravels a defect of the suppressor and provides direct proof for a functional equilibrium between TFIIA and NC2. It does

not necessarily illuminate the physiological role of NC2. In fact, yeast carrying the suppressor is viable in the absence of NC2. Thus, it should not be critical whether or not mt-Toa1 relieves repression by NC2. The mt-Toa1 should rather be defective in a stimulatory function, such as seen in the ternary complex formation. However, there are many other players in the cell that will sense the differences in recruitment activities with respect to TBP and stability of the resulting complex. One of these components is probably the RNA polymerase II holoenzyme (Kim *et al.*, 1994; Koleske and Young, 1994). In line with this hypothesis, another suppressor of NC2 that was isolated was shown to carry mutations in *sin4* (M.Lemaire, J.Xie, M.Meisterernst and M.Collart, in preparation). Hence, the fact that the suppressor shows deficiencies in ternary complex formation might be considered as an indication that lack of NC2 creates a demand for a changed control on this trigger.

Materials and methods

Plasmids, strains and media

Yeast strains are indicated in Table I. Growth media were as described (Ausubel *et al.*, 1990). pMAC102, a pPC62 (Chevray and Nathans, 1992) derivative is obtained by *Hind*III digestion followed by religation, containing *ADH1* promoter and *ADH1*-3'-untranslated sequences. pJX14 (Table II) is derived from pMAC102 and pRS424 (Pitulle *et al.*, 1992). pJX14 is a high-copy plasmid that also contains *ADH1* promoter and *ADH1*-3'-untranslated sequences and *TRP1* marker. Wild-type hNC2 β and deletion mutants were cloned into the multiple cloning sites (MCS) between the *ADH1* promoter and 3'-untranslated sequences of pJX14 creating pJX8, pJX9, pJX20 and pJX26 (see Table II), whereas wt-hNC2 α , wt-hNC2 β and the hNC2 α deletion mutants were cloned into MCS of the low-copy vector pMAC102 (pJX1, pJX3 to pJX6 and pJX23, see Table II). The suppressor mutation was isolated by PCR from the strain MY1802 using the following oligonucleotides: forward primer, 5'-GATTGAATTCTGCTCGCGTTGCTGAGC-3' and reverse primer, 5'-GATTGGATCCTTTTATAATTTGACAGATGAG-3', and successively cloned into the pBS SK(+) vector (Stratagene) obtaining pML83 (Table II). For the expression of recombinant proteins, cDNA of wt-Toa1 and Toa2 genes were cloned in pET vectors (Novagen). cDNA of the mt-Toa1 strain was isolated from pML83 by PCR using oligonucleotides: forward primer, 5'-GACCAAGTTCGTTGCATCCCTCTTTATTTC-3' and reverse primer, 5'-TGAACCATTAAGAGGAACTCATCGTACAC-3'. The PCR product was cloned into a pET vector containing His₆ tags (Novagen), obtaining pJX27.

Expression and purification of proteins

Purification of hTBP and yTBP was performed as described previously (Kretzschmar *et al.*, 1994; Stelzer *et al.*, 1994; Kaiser *et al.*, 1995). Recombinant hNC2 α and hNC2 β were expressed and purified as described by Goppelt *et al.* (1996). wt-Toa1, mt-Toa1 and Toa2 were overexpressed in *E.coli* and purified from inclusion bodies under denaturing conditions. Equal amounts of wt-Toa1 and Toa2 or mt-Toa1 and Toa2 were combined in buffer A containing 20 mM Tris–HCl (pH 7.3 at room temperature; RT), 0.2 mM EDTA pH 8.0, 20% glycerol, 100 mM KCl and 8 M urea was added. Renaturation was initiated through dialysis against buffer A containing 4 M urea at RT for 6 h, followed by a dialysis against buffer A–2 M urea performed overnight, followed by dialysis against buffer A in the absence of urea for 12 h at 6°C. Active yTFIIA concentrations were estimated from titrations in the presence of an excess of TBP and DNA in gel shift experiments.

Suppressor screen

A centromeric plasmid carrying the hNC2 β gene and *LEU2* marker (pJX3) was transformed into MY1780 and transformants were then transferred onto FOA plates. Slow growing Leu⁺ colonies were obtained (JY20). Eight different colonies were grown in liquid rich media (YPD) at 30°C to exponential phase or to saturation stage (2–3 days). From the liquid culture, 10⁷ cells were streaked out on YPD plates and incubated at 30°C. After 2–3 days suppressors were selected. One of the suppressors (MY1802) was further characterized. It displayed a cold-sensitive

phenotype at 16°C and grew similarly to wild-type yeast strains at 30 and 37°C. The suppressor phenotype was not caused by the *CEN* plasmid carrying the hNC2 β gene, as could be verified by sequencing the plasmid rescued from MY1802. This suppressor was crossed with a strain isogenic to JY20 but of opposite mating type (MY1855). Tetrad analysis revealed that the suppressor phenotype segregated 2:2 for cold sensitivity and faster growth. MY1802 was crossed with its parental wild-type strain (MY4). Tetrads were dissected and spores without the NC2 β gene were obtained. They carried G418 resistance and had a Leu⁻ phenotype. A heterozygous diploid strain with disruption of one copy of the yNC2 α gene was generated by substituting one copy of the yNC2 α gene with a gene conferring G418 resistance (MLY31, Table I). MLY31 was transformed with a *URA3* plasmid carrying the yNC2 α gene. Ura⁺ transformants were sporulated, tetrads dissected and cells carrying a disruption of the genomic yNC2 α gene and yNC2 α gene on *URA3* plasmid were obtained (MY1784). MY1784 was crossed to MY1870, and tetrad analysis was performed. All G418 resistant spores obtained were Ura⁺, some were additionally cold sensitive. The diploid strain (MLY462, Table I) carrying the *Toa1* gene disruption was generated by replacing the *Clal-SpeI* fragment of the *Toa1* gene with a *Clal-SpeI* cassette containing the *URA3* gene. MLY462 was transformed with a plasmid carrying the mt-*Toa1* allele (pML83), transformants were sporulated, dissected and the haploid strain MLY465 was obtained. MLY465 was crossed to MY1781 (Table I) and a strain carrying the yNC2 β - and the *Toa1* gene disruptions (MLY504) was recovered.

Electrophoretic mobility shift assay (EMSA)

Purification of hTBP and yTBP was performed as described previously (Kretzschmar *et al.*, 1994; Stelzer *et al.*, 1994; Kaiser *et al.*, 1995). Active recombinant yTFIIA (1–50 ng) and human or yeast TBP (5–50 ng total, as detailed in the figure legends) were incubated with 75 fmol of a Klenow-labelled 60 bp HIV core promoter oligonucleotide (sequence in Kaiser *et al.*, 1995) for 30 min at 28°C in a buffer containing 5 mM MgCl₂, 25 mM HEPES–KOH pH 8.2, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.025 μ g/ μ l poly(dG–dC), 0.2 mg/ml bovine serum albumin (BSA), 60–80 mM KCl and 8% (v/v) glycerol. Reaction mixtures were separated on a 5% PAA (37.5:1 acrylamide to bisacrylamide) gels containing 1.5 mM MgCl₂ and 1 mM DTT in 1 $\times$ TGE buffer containing 1.5 mM MgCl₂. Gels were pre-run for 2 h at 140 V and electrophoresis was carried out at 6°C for 6 h at 140 V.

DNase I footprint analysis

Recombinant human or yeast TBP (50 ng) and yTFIIA proteins at the concentrations indicated were incubated with 50 fmol of a ³²P-labelled HIV core promoter fragment either 60 or 90 bp in length, (sequences in Kaiser *et al.*, 1995) under standard EMSA conditions. Following incubation at 28°C for 30 min, reactions were diluted 1:3 with DNase I buffer containing 5 mM CaCl₂, 50 mM KCl, 10 mM HEPES–KOH pH 8.2, 0.2 mg/ml BSA and 50 μ g/ml poly(dG–dC) and incubated at RT for 1 min. DNase I (0.4 μ g/ml) was added, reactions were incubated at RT for 1 min and subsequently stopped with 3 vol of a buffer containing 10 mM Tris–HCl pH 7.8 at RT, 0.1 M LiCl, 0.5% SDS, 7 M urea, 10 mM EDTA pH 8.0, 0.3 M NaOAc pH 5.3 and 0.2 mg/ml tRNA, phenolized and ethanol precipitated. (A+G) Maxam and Gilbert reactions were conducted according to a protocol of Bencini *et al.* (1984). Samples were analysed on 10% (19:1 acrylamide to bisacrylamide) (90 bp fragment) and 13% (19:1 acrylamide to bisacrylamide) (60 bp fragment) denaturing polyacrylamide gels, respectively.

In vitro transcription

In vitro transcription reactions were carried out in a partially purified system containing the 0.5 M KCl fraction (4 μ g protein) of HeLa nuclear extracts fractionated according to standard procedures on phosphocellulose (P11, Meisterernst *et al.*, 1991), recombinant purified TFIIIB (20 ng) and hTBP (16 ng), and the amounts of recombinant *E.coli*-expressed and purified human NC2 and yTFIIA as indicated in the figure legends. Transcription reactions were conducted with supercoiled HIV core-, pMRG5 (Kretzschmar *et al.*, 1994), and ML promoters, pMLA53 (Meisterernst *et al.*, 1991), located upstream of G-less cassettes. Reactions were stopped with 20 vol of a buffer containing 10 mM Tris–HCl pH 7.8 at RT, 0.1 M LiCl, 0.5% SDS, 7 M urea, 10 mM EDTA pH 8.0, 0.3 M NaOAc pH 5.3 and 0.2 mg/ml tRNA, phenolized with 1 vol of phenol/chloroform/isoamylalcohol and precipitated with 1 vol of isopropanol. RNA transcripts were analysed on 5% (19:1 acrylamide:bisacrylamide) denaturing polyacrylamide gels.

Yeast extracts

Fresh overnight culture of yeast strain with wt-yTFIIA (MY1) and strain carrying mt-yTFIIA (MY1870) were diluted to OD₆₀₀ 0.3 in liquid YPD media and grown at 30°C to an OD₆₀₀ of 0.8–1.0. The cells were harvested, washed and suspended in 10 vol of buffer FA-0.1 containing 25 mM HEPES–KOH pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol and 30 mM DTT. After incubation at RT for 30 min, cells were collected and pellets were resuspended in 2 vol of buffer FA-0.1 containing 1 mM DTT and zymolyase. Cell suspensions were incubated for 30 min at 37°C until they appeared clumpy. Total cell lysates were prepared by adding 1/5 vol of buffer FA 2.5 (25 mM HEPES–KOH pH 7.5, 2.5 M NaCl, 1 mM EDTA, 10% glycerol and 1 mM DTT). The cell debris was removed by centrifugation at 2000 r.p.m. at 4°C for 20 min, supernatants were pooled and directly applied to gel filtration columns.

Gel filtration chromatography and Western blotting

Renatured wt- or mt-yTFIIA complex (5 μ g) in buffer A containing 100 mM KCl was loaded onto Superdex 75 PC 3.2/30 column (SMART-Pharmacia) equilibrated in buffer A. Fractions (100 μ l) were collected and analysed by SDS–PAGE. For gel filtration experiments with native yTFIIA, 250 μ g of total extracts from yeast strains expressing either wt- or mt-yTFIIA were applied onto a Superdex 75 column. One-hundred-microlitre fractions were collected and analysed by Western blotting using anti-Toa2 antibody and standard procedures.

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