Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Molekulare Funktion und Regulation des negativen Cofaktors 2, NC2

Elisa Piaia
aus
Montebelluna, Italien
2005

Erklärung

Diese Dissertation wurde im Sinne von §13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von PD Dr. Michael Meisterernst betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, 23.11.2004

Elisa Piaia

Dissertation eingereicht am: 23.11.2004

1. Gutachter: PD Dr. Michael Meisterernst

2. Gutachter: Prof. Dr. Patrick Cramer

Mündliche Prüfung am: 15.03.2005

Molecular function and regulation of the negative cofactor 2, NC2

a Lino

TABLE OF CONTENTS

SUMMARY

Initiation of transcription by eukaryotic RNA polymerase II is finely controlled by a multitude of regulatory factors. Among them, the negative cofactor 2 (NC2), composed of the subunits NC2α and NC2β, is able to bind directly to TBP-DNA complexes, preventing the assembly of the general transcription factors TFIIA and TFIIB. Despite extensive research on the negative and positive function of NC2, several questions concerning its regulation remain unexplored. In particular, localization and post-translational modifications are poorly understood.

This work is the first to give some insights on the regulation of this factor.

We present evidence that both subunits contain a nuclear localization signal (NLS) responsible for the accumulation of proteins in the nucleus. Immunofluorescence studies showed that NC2 dimer localizes exclusively in the nucleoplasm. However, the two subunits reveal characteristic and unique distribution patterns: NC2α is also found in the nucleoli, and NC2β in small concentrations also in the cytoplasm. Moreover, we show that the two subunits already dimerize in the cytoplasm and are transported into the nucleus as a complex. Interestingly, both NLS are essential for import of the dimer.

We also report for the first time several isoforms of both subunits. *In vivo* labeling experiments showed that NC2α is specifically hyperphosphorylated during mitosis. This modification does not impair its ability to dimerize with the partner and bind to TBP-DNA complexes, nor affects the stability of the complex. Furthermore, the phosphorylated protein maintains the ability to mobilize TBP on the DNA. These results suggest that NC2 is still bound to DNA during mitosis, in line with the idea that this factor keeps TBP stably associated to DNA.

I. INTRODUCTION

1. Eukaryotic gene expression: from gene sequence to active protein

The process of gene expression involves the whole series of events that, starting from transcription of a gene, leads to the synthesis of a functional protein. During the transcription process, the information is transferred from the deoxyribonucleic acid (DNA) to the messenger ribonucleic acid (mRNA). The enzyme responsible for this reaction is called RNA polymerase (RNA Polymerase II in the eukaryotes, Pol II), which is aided in its function by other factors, termed general transcription factors (GTFs).

In most of the genes the level of expression is regulated by transcription factors that bind directly to regulatory DNA sequences. The interaction of transcription activators with different kinds of coregulatory proteins promotes chromatin decompaction and access to DNA, facilitating recruitment of the RNA transcription machinery at the transcription start site. As soon as the nascent RNA is transcribed, the 5' end of the molecule is modified by the addition of a "cap". During transcription elongation, the polymerase moves from the 5' to the 3' end of the gene sequence to extend the transcript. Both coding regions (exons) and non-coding regions of the gene (introns) are transcribed by the Pol II in the pre-mRNA transcript. At the same time, the splicing machinery starts to remove the introns from the pre-mRNA, and the proteins involved in export of the mature mRNA bind to the molecule. Upon reaching the end of a gene, Pol II stops transcribing (termination), the newly synthesized RNA is cleaved, and a polyadenosine tail [(polyA)] is added to the 3' end of the transcript. The newly synthesized mRNA is then transported from the nucleus to the cytoplasm, where the information is transferred from the mRNA to the protein (translation). In eukaryotes, the nuclear membrane separates the compartments where transcription and translation take place, and the export of the protein-bound mRNA is mediated by apposing pore structures associated to the nuclear envelope. Once in the cytoplasm, translation of mRNA takes place on large ribonucleprotein complexes called ribosomes. The newly synthesized protein undergoes folding and, eventually, post-translational modifications.

The traditional view of gene expression considered each of the steps in the pathway from gene to protein (transcription-mRNA-processing-export-translation), as independent and chronologically subsequent events. Yet, in recent years this view has changed, and now the diverse stages are considered physically and functionally related to one another, so that different processings are overlapping rather then distinct (Orphanides and Reinberg 2002). As a consequence, the gene expression pathway is regulated at multiple levels, since each process is controlled *per se* and also in coordination with the others. Increasing observations point to the notion that the passage from transcription initiation to transcription elongation is not only dependent on the accurate work of transcription factors and polymerase, but also on the capping machinery related to mRNA processing and mRNA export (Proudfoot et al. 2002; Meinhart and Cramer 2004).

2. Promoter structure in eukaryotic class II genes

Depending on the enzyme responsible for their transcription, eukaryotic genes have been divided in three classes. RNA Pol I transcribes the ribosomal RNAs (rRNAs), which are components of the ribosomes. Transcription of protein-coding genes is catalyzed by RNA Pol II, whereas RNA Pol III is involved in synthesis of the 5S rRNA and the transfer-RNAs (tRNAs).

2.1. The core promoter elements

The core promoter of the class II genes is defined as the minimal DNA sequence required for correct positioning and assembly of the pre-initiation complex (PIC), which is composed by the RNA Pol II and the general transcription factors (GTFs). Characteristic core promoter elements are the TATA box, the Initiator (Inr), the TFIIB recognition element (BRE), located directly upstream the TATA box and, in Drosophila genes, the downstream promoter element (DPE) element. Most promoters contain one or more of these elements, but no one is essential for promoter function. Promoter elements are binding sites for subunits of the PIC and serve to orient the transcription machinery in order to direct unidirectional transcription.

2.1.1. TATA-box

The TATA sequence is the binding site for the TATA binding protein (TBP). TATA elements in *Saccaromices cerevisiae* are typically located 40 to 120 bp upstream of the transcription start site, but in other eukaryotes, including *Schizosaccharomyces*

pombe, it is usually located at a fixed distance of 25 to 30 bp from the start site (Struhl 1995). Functional analysis of the mutated sequence defined the sequence TATAAA as the consensus TATA sequence in yeast (Chen and Struhl 1988; Singer et al. 1990; Wobbe and Struhl 1990). Yeast and human TBP have nearly identical TATA sequence requirements, emphasizing the evolutionary conservation of the TBP-TATA interaction. In yeast, transcription from TATA-less promoters remains TBP dependent, and presumably, other components of the transcription machinery recognize a promoter structure other than TATA to nucleate PIC assembly, like the Inr element or the DPE.

2.1.2. Initiator Elements

Inr elements are DNA sequences encompassing transcription start sites. The position of the Inr is not defined by its distance with respect to the TATA box, since this distance is not constant in all organisms. Rather, TATA elements define the window within which initiation can occur. Mutational analyses have defined preferred Inr sequences (Furter-Graves and Hall 1990), but there is not a clear consensus sequence, yet.

2.1.3. DPE

The DPE is located at ca. 30bp downstream from the transcription start site and seems to act, in conjunction with the Inr element, as a TFIID binding site for the TAF6 and TAF9 at TATA-less promoters (Butler and Kadonaga 2002; Kadonaga 2002).

2.2. Regulatory sequences

2.2.1. Enhancer and Silencer elements

In metazoans enhancer and silencer elements are DNA sequences that function as binding sites for transcriptional activators and repressors, respectively. They function in either orientation and independently from their distance to the core promoter (up to many kilobasepairs), being found both upstream and downstream of the TATA box and also in the intron regions of genes. Upstream activation sequences (UAS) are the yeast analogs of the enhancers, with the difference that they do not function when positioned downstream of the TATA box (Guarente and Hoar 1984; Struhl 1984).

Once associated with the cognate enhancer elements, transcriptional activators facilitate the assembly of the PIC, either directly contacting the GTFs, or indirectly through coactivators. Vice versa, binding of repressors to the silencer elements

recruits remodeling complexes that promote chromatin compacting.

2.2.2. Insulator

Insulators are complex DNA sequence elements that can help to preserve the independent function of genes embedded in a genome in which they are surrounded by regulatory signals they must ignore (Burgess-Beusse et al. 2002; Kuhn and Geyer 2003). In some cases insulators can serve as barriers to protect a gene against the encroachment of adjacent inactive condensed chromatin. Some insulators also can act as blocking elements to protect against the activating influence of distal enhancers associated with other genes. In that case, insulators prevent enhancer action when placed between enhancer and promoter, but not otherwise. Although most of the insulators identified so far derive from Drosophila, they also are found in vertebrates (Burgess-Beusse et al. 2002).

3. The preinitiation complex: the general transcription factors and the RNA Polymerase II

3.1. The general transcription factors (GTFs)

Pioneering studies performed in Roeder laboratories showed that purified mammalian RNA Pol II was able to initiate RNA synthesis from template DNA only when supplemented with a crude cell extract (Weil et al. 1979). Different fractions of extracts were tested for the ability to allow *in vitro* RNA Pol II transcription activity, leading to the identification of the general transcription factors (GTFs) (Matsui et al. 1980). Unlike the prokaryotic enzymes, eukaryotic RNA polymerases alone cannot recognize the promoters of their target genes, and instead rely on a series of accessory factors, the GTFs.

The GTFs include TBP, TFIIB, TFIIE, TFIIF and TFIIH, and were identified biochemically as factors required for accurate transcription initiation by RNA Pol II from double stranded DNA templates *in vitro* (Roeder 1996; Orphanides and Reinberg 2002).

Biochemical approaches investigating preinitiation complex formation demonstrated that PIC assembly is nucleated *in vitro* by binding of TBP, followed by the concerted recruitment of TFIIB, RNA Pol II-TFIIF, TFIIE, and TFIIH (see Fig. 1; Van Dyke et al. 1988; Buratowski et al. 1989; Maldonado et al. 1990)

3.1.1. TBP

TBP was identified as part of TFIID, a multiprotein complex of ca. 700kD in size, composed also of 14 TBP-associated factors (TAFs) (Albright and Tjian 2000; Tora 2002). TBP is a universal transcription factor, required for initiation by all three eukaryotic RNA polymerases (Hernandez 1993), by association with a distinct set of factors. In metazoans, four different TBP complexes have been described: the transcription factors SL1 (RNA Pol I), TFIID (RNA Pol II), TFIIIB (RNA Pol III) and $SNAP_c$, another RNA Pol III-TBP complex required for transcription of certain small nuclear RNA (snRNA) genes.

In human TBP, two different domains can be distinguished: a core domain, composed of two imperfect repeats highly conserved among eukaryotes, and an N-terminal domain, containing a glutamine rich region conserved among vertebrates, but not present in yeast. Crystallographic studies of the core domain of TBP bound to a TATA box revealed that TBP has a saddle-like structure (Burley and Roeder 1996). Unlike other DNA binding proteins, the concave surface of the TBP saddle makes contacts with the minor groove of the DNA (Lee et al. 1991; Starr and Hawley 1991; Kim et al. 1993a; Kim et al. 1993b). TBP saddle binds the 8-bp TATA element, unwinding about a third of a helical turn and bending the DNA 80° toward the major groove (Kim et al. 1993a; Kim et al. 1993b). Although the TBP molecule is symmetrically shaped, the protein surface of the two repeats is very divergent, forming a large asymmetric protein-DNA interface, thus creating a platform for binding other components of the transcription machinery. TBP does not bind to TATA elements with high orientation specificity (Cox et al. 1997). Other promoter elements in combination with TATA determine instead the orientation of transcription machinery assembly at a promoter (Inr, BRE, DPE).

3.1.1.1. TBP paralogues

Database searches for paralogous genes encoding for the GTFs revealed that TFIIB, TFIIE, TFIIF, TFIIH, and Pol II are encoded by single-copy genes (Aoyagi and Wassarman 2000). On the contrary, both TBP and TAFs have paralogue genes. Concerning TBP, at least two new gene families encoding proteins related to TBP have been found in metazoans. To one group belong the TBP-like factor (TLF), or TBP-related factor 2 (TRF2), identified in C. elegans, Drosophila and vertebrate. Unlike TBP, TLFs may bind to DNA sequences other than canonical TATA box (Dantonel et al. 1999; Rabenstein et al. 1999). In fact, vertebrate TLFs support transcription from a TATA-less promoter (Ohbayashi et al. 2003). *D. melanogaster* also contains a further TBP-related factor called TRF1. A second group of TBP paralogue genes, called TBP2s, has been recently identified, also in vertebrates

(Veenstra and Wolffe 2001). Binding of vertebrate TBP2 to TATA box promoters has not yet been documented.

It is likely that higher organisms have developed different TBP-like factors to allow differential gene expression according to the developmental stage and the differentiation state of the cells. In line with this hypothesis, also TAFs have paralogous genes and the TAF-like proteins are usually expressed in a cell type and tissue-specific pattern.

3.1.2. TFIIB

TFIIB is recruited to the PIC after TBP. TFIIB interacts directly with the DNA, where it recognizes the BRE element (Imbalzano et al. 1994), with TBP and RNA Pol II, as well as other GTFs (Goodrich et al. 1993; Ha et al. 1993; Chen and Hampsey 2004). Thus, TFIIB plays a role in the correct selection of the transcription start site (Hawkes and Roberts 1999), its recruitment is a prerequisite for binding of the RNA Pol II to the PIC (Buratowski et al. 1989), and it is also a target of many gene-specific transcriptional activators (Lin et al. 1991).

3.1.3. TFIIF

TFIIF has two subunits, called RAP30 and RAP74 in human, conserved among eukaryotes.

TFIIF binds tightly to RNA Pol II, suppresses non-specific DNA binding of RNA Pol II and stabilizes the PIC (Conaway and Conaway 1993). TFIIF is also implicated in start site selection. For these reasons, this transcription factor is thought to be the eukaryotic correspondent of the bacterial σ factor. In fact, both subunits show limited sequence similarity to σ factor (McCracken and Greenblatt 1991; Garrett et al. 1992). TFIIF is also important during elongation, since it prevents pausing of RNA Pol II (Bengal et al. 1991). The RAP74 subunit is phosphorylated by TAF1 (Dikstein et al. 1996). Moreover, TFIIF itself has been shown to posses an associated ser/thr kinase activity, which may modulate its activity (Rossignol et al. 1999).

3.1.4. TFIIE

TFIIE enters the PIC after RNA Pol II and prior to TFIIH (Buratowski et al. 1989). TFIIE interacts directly with the unphosphorylated form of RNA Pol II, TFIIF (both subunits) and TFIIH (Flores et al. 1989). TFIIE is also a target of gene-specific transcription activators (Sauer et al. 1995; Zhu and Kuziora 1996). Functions attributed to this factor include recruitment of TFIIH to the PIC (Flores et al. 1992), stimulation of both the kinase and helicase activities of TFIIH (Ohkuma 1997; Lee and Young 2000). Structure-function analysis suggests that TFIIE could act as a

checkpoint for formation of the PIC via its control of TFIIH recruitment and activities (Ohkuma et al. 1995). Some promoters are more dependent on TFIIE than others, in particular TATA-containing promoters (Sakurai et al. 1997).

3.1.5. TFIIH

The last factor to bind the PIC is TFIIH. TFIIH is composed of nine polypeptides with four enzymatic activities, comprising DNA-dependent ATP-ase (Roy et al. 1994), two ATP-dependent DNA helicases (Schaeffer et al. 1993), and CTD kinase (Lu et al. 1992). TFIIH is an essential factor in transcription, as well as in nucleotide excision repair (NER), and has also been implicated in mammalian cell cycle progression. Consistent with these multiple roles, TFIIH is also the most complex GTF. TFIIH exists in two forms: (1) a six subunit core complex, containing the two DNA helicase activities and active in nucleotide excision repair (Svejstrup et al. 1995); and (2) the holo-TFIIH, that results from the association of a core, containing the two DNA helicase activities, and a kinase domain termed CAK (CDK-activating kinase), containing Cdk7 (Coin and Egly 1998). The activity and substrate specificity of Cdk7 are regulated by phosphorylation and / or association with other polypeptides like Mat1 and core TFIIH (Busso et al. 2000). The ADP-dependent DNA helicase activity of the XPB subunit is required for formation of an open promoter complex (promoter melting) (Wang et al. 1992a; Holstege et al. 1996; Moreland et al. 1999), whereas the CTD kinase activity is implicated in the transition from transcription initiation to elongation (promoter clearance) (Dvir et al. 1997). Furthermore, TFIIH promotes transition from very early elongation complexes to stable elongation complexes (Dvir et al. 1997).

The dual TFIIH role in transcription and DNA repair seems to correlate with the observation that transcriptionally active genes are preferentially repaired (Mellon and Hanawalt 1989).

3.2. RNA Polymerase II

RNA Polymerase II (Pol II) is a multiprotein complex composed of 12 subunits with a total size of 500 kD. Subunits of RNA Pol II, in particular the two largest ones, Rpb1 and Rpb2, are highly conserved among eukaryotes. These subunits can be classified in three categories: (i) subunits of the core domain, having homologous counterparts in RNA Pol I and III (Rpb1, 2, 3, 11), (ii) subunits shared between all three nuclear polymerases (Rpb5, 6, 8, 10 and 12), and (iii) subunits specific to Pol II, but not essential for transcription elongation (Rpb4, 7and 9) (Cramer et al. 2000; Cramer et al. 2001).

3.2.1. CTD

A unique feature of the largest RNA Pol II subunit, Rpb1, is the presence of tandem repeats of a heptapeptide sequence at its carboxy-terminal domain (CTD). The sequence of the repeat is Tyr-Ser-Pro-Thr-Ser-Pro-Ser, with both Ser2 and Ser5 as sites of phosphorylation. Although the presence of the CTD is a ubiquitous feature among eukaryotic RNA Pol II, the repeat length varies and increases with increasing genome complexity (26 or 27 repeats in yeast, 34 repeats in *C. elegans*, 43 in *D. melanogaster*, 52 in human). Depending on the phosphorylation state of the CTD, two forms of RNA Pol II exist *in vivo*: IIA, which is not phosphorylated, and IIO, which is extensively phosphorylated. The IIA form preferentially enters the PIC (Lu et al. 1991), whereas IIO is present in the elongation complex (Dahmus 1996). Conversion of IIA to IIO occurs concomitant with, or shortly after, the transition from initiation to elongation (O'Brien et al. 1994).

3.2.2. The transcription cycle

In the pre-initiation complex, Pol II and the general transcription factors are all bound to the promoter, but are not in an active conformation to begin transcription (Fig. 1). Next, a marked conformational change occurs, in which 11-15 base pairs (bp) of double strand DNA surrounding the transcription start site are melted and the template strand is positioned within the active site cleft of Pol II to form the open complex (Wang et al. 1992a). Pol II is unique among cellular Pols in requiring the action of an ATP-dependent DNA helicase (XPB) for open complex formation. In many systems, multiple short RNAs (from three to ten bases), termed abortive products, are synthesized before Pol II productively initiates synthesis of full-length RNAs (Luse and Jacob 1987; Holstege et al. 1996). After synthesis of ca. 30 bases of RNA, Pol II is thought to release its contacts with the core promoter and the rest of the transcription machinery (promoter clearance), and to finally enter the stage of transcription elongation. The transition from transcription initiation to transcription elongation is accompanied by phosphorylation of the CTD of Pol II. Different kinases are involved in CTD phosphorylation. During promoter melting, CTD is phosphorylated at Ser5 by two cyclin dependent kinases: Cdk7, a subunit of the general transcription factor TFIIH, and Cdk8 (Lee and Young 2000; Liu et al. 2004). Although previous work indicated that only Cdk7 positively regulates transcription, recent work indicates that in yeast both kinases can promote transcription *in vivo* and *in vitro* (Liu et al. 2004). After initiation, other kinases such as Cdk9/Ctk1 phosphorylate Ser2, resulting in recruitment of the RNA processing, polyadenylation and termination factors to the elongating RNA Pol II. The flexibility

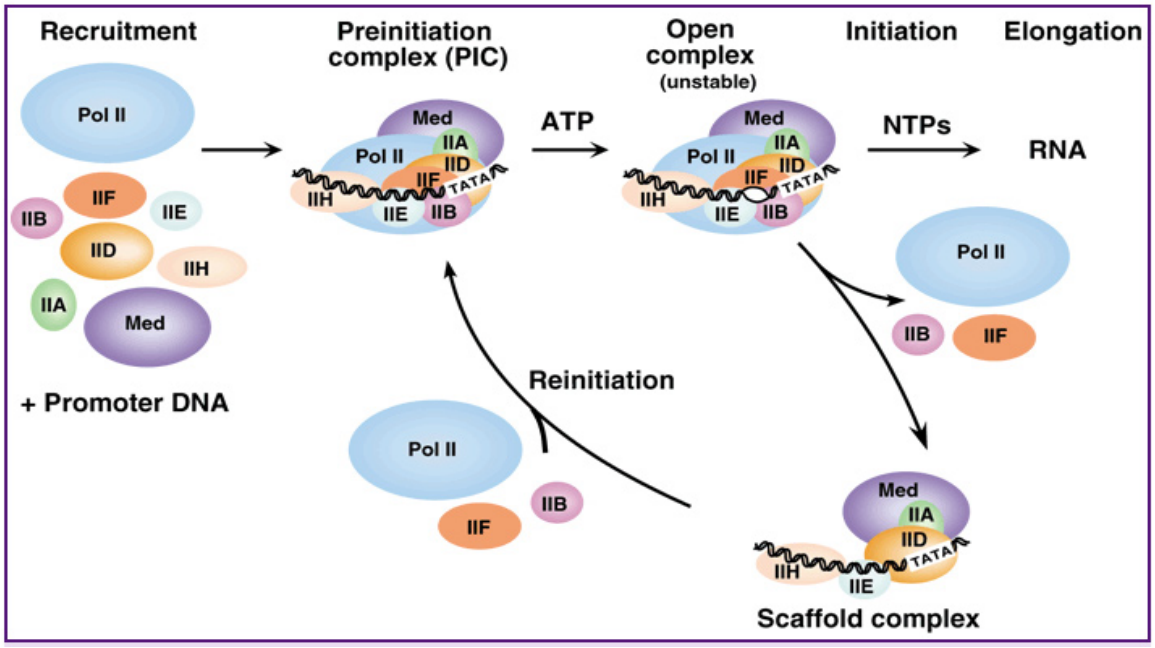


Figure 1. The pathway of transcription initiation and reinitiation for RNA Polymerase II. For abbreviations, see text. From Hahn (2004).

of the CTD tail combined with multiple modifications by phosphorylation, makes Pol II a central player in regulation of transcription initiation and in coordinating transcription to mRNA processing events (Hirose and Manley 2000; Bentley 2002; Proudfoot et al. 2002; Meinhart and Cramer 2004).

After initiation of transcription, Pol II and the general transcription factors TFIIB and TFIIF, dissociate from the promoter, leaving the remaining general factors at the promoter in the Scaffold complex (Yudkovsky et al. 2000; Hahn 2004). This complex presumably marks genes that have been transcribed and enables the typically slow step of recruitment to be bypassed in subsequent rounds of transcription. In order to be reincorporated in a new initiation process, the phosphatase Fcp1 recycles Pol II to its unphosphorylated form, allowing the GTFs and Pol II to initiate another round of transcription (Reinberg et al. 1998; Cho et al. 2001).

3.2.3. The holoenzyme

Biochemical approaches investigating preinintiation complex formation in a reconstituted *in vitro* system suggested that RNA Pol II and GTFs assemble to the promoter in a defined and sequential order (Van Dyke et al. 1988; Buratowski et al. 1989; Maldonado et al. 1990). This scenario was challenged by the purification of preassembled complex, containing GTFs other than TBP, Pol II and other regulatory factors (Mediator), first in yeast (Chao et al. 1996; Chang and Jaehning 1997) and

later also in human cells (Ossipow et al. 1995; Maldonado et al. 1996; Chang and Jaehning 1997). These findings suggested that Pol II and its accessory factors are recruited to the promoter as a preformed complex, in the form of a “holoenzyme” (Conaway and Conaway 1993; Ossipow et al. 1995). However, the exact composition of RNA Pol II holoenzymes is not known *in vivo* and certain holoenzyme components are recruited to some promoters independently of Pol II (Cosma et al. 1999; Ranish et al. 1999; Rani et al. 2004). Moreover, PICs are composed of different factors at distinct promoters (Muller and Tora 2004). Furthermore, the relative abundance of yeast GTFs is more compatible with a step-wise assembly model (Borggrefe et al. 2001). Hence, whether PIC assembly *in vivo* occurs in a stepwise manner or by holoenzyme recruitment is still not completely clear.

4. Transcriptional activators and repressors

The complexity of gene regulation is mirrored by the high number of genes coding transcription factors (*e.g.* Tupler et al. 2001). Transcription factors, binding to promoters or enhancer-regions, can stimulate or repress gene transcription. Usually, they consist of two domains: (i) a DNA binding and (ii) an activation (or repression) one. Typical DNA binding domains in eukaryotes are the basic helix-loop-helix and leucine zipper, zinc finger, and homeodomain. Present in at least one copy, they interact with the major grooves of the correspondent cognate site in the DNA. Most genes are under the control of multiple transcription factors that attach to the same transcription-control regions. Moreover, cofactors (coactivators or corepressors) often coregulate transcription by interaction with transcription factors. This means that frequently the real effect of a transcription factor depends on which cofactor it binds with (Kaiser and Meisterernst 1996; Lemon and Tjian 2000; Malik and Roeder 2000; Courey and Jia 2001; Naar et al. 2001).

5. Transcriptional cofactors/coregulators

Transcriptional cofactors function as adaptor molecules between sequence specific DNA binding activators or repressors and the transcriptional machinery (Burley and Roeder 1996; Kaiser and Meisterernst 1996).

Coactivators are distinct from GTFs, since they are not dispensable for basal transcription *in vitro*. They are also distinct from activator in that most do not directly bind DNA and none binds DNA in a sequence specific manner. Coactivators

interact either with components of the core transcriptional machinery (TFIIA, TAFs, USA (upstream stimulatory activity) factors, SRB/Mediator), or with nucleosomes, promoting chromatin modification (HATs), or chromatin remodeling (SWI/SNF). Moreover, other gene or cell type specific coactivators have been described in metazoan systems (Fondell et al. 1996; Kim et al. 1996).

5.1. TAFs: TBP associated factors

Whereas TBP is required for promoter recognition and assembly of the other GTFs in the PIC (Buratowski et al. 1988), activation of transcription is observed in *in vitro* systems only when TFIID is part of the transcription machinery (Pugh and Tjian 1990; Burley and Roeder 1996). This observation led to the discovery of the TAFs and to the idea that TAFs function in transcriptional activation by relaying information from activators to the core transcriptional machinery. Functions attributed to TAFs are: coactivator activity, facilitating promoter recognition, enzymatic activities. TAFs seem to function as coactivators in a gene-specific manner, since they are not generally required for activation of yeast genes (Moqtaderi et al. 1996; Walker et al. 1996), but rather of specific genes, including those involved in cell cycle progression (Apone et al. 1996; Walker et al. 1997).
The TAF1, the biggest TAF, (TAFs are named after the new nomenclature, Tora 2002) has three enzymatic activities: phosphorylation of the RAP74 subunit of TFIIF (Dikstein et al. 1996), histoneacetyltransferse (HAT) and ubiquitinligase modification of Histone H1 (Mizzen et al. 1996; Imhof et al. 1997).
TAF2 shows specific DNA binding activities in the region immediately downstream of TATA box up to the initiator (Verrijzer et al. 1994), whereas TAF6 and TAF9 bind the DPE element of Drosophila (Burke and Kadonaga 1997). Probably the cooperative binding of several TAFs subunits facilitates promoter recognition and enhances stability of TFIID-DNA complex (Albright and Tjian 2000).
Sequence analysis of TAFs reveals similarity with the histones (Burley and Roeder 1996). In fact, many TAFs contain the characteristic histone fold domain through which they can make pairs similar to the histones H2A-H2A.

5.1.1. TAF paralogues

Depending on TAF composition, many different TFIID complexes have been isolated from human cells, with differences regarding the composition of the core TAFs, as TAF10-lacking hTFIID (Brou et al. 1993; Jacq et al. 1994) and TAF6δ-containing TAF9-lacking complex (Bell et al. 2001); or of the cell-type specific TAFs, as B cells

and ovary-specific hTFIID containing TAF4b (Dikstein et al. 1996; Freiman et al. 2001) and hTFIID involved in spermatogenesis, containing TAF1L and TAF7L.
The existence of different TFIID complexes could reflect different roles in recognition of distinct promoters, in mediating response to different activator/coactivators, and differential regulation based on the interaction with distinct transcription factors.

5.2. TFIIA

Since TFIIA is not absolutely required for PIC assembly and subsequent transcription in a purified *in vitro* transcription system, it is not considered a general transcription factor. Human and Drosophila TFIIA contains three subunits. In both organisms, the two largest subunits are coded by the same gene and derived from posttranslational modifications of the same precursor protein (DeJong and Roeder 1993; Yokomori et al. 1993). The yeast homologue has two polypeptides, encoded by the *TOA1* and *TOA2* genes
TFIIA binds TBP (Buratowski et al. 1989), is located upstream of TATA, stabilizes TBP-TATA box interaction (Imbalzano et al. 1994) and contributes to the formation of an open promoter complex (Wang et al. 1992b). TFIIA also interacts with specific transcriptional activators, TAF4 (formerly $TAF_{II}110$), the coactivators PC4 and HMG2 and is considered to stimulate transcription through displacement of transcriptional repressors such as NC2, PC3/Dr2 (topoisomerase I), HMG1 and Mot1 from the TFIID complex (Meisterernst and Roeder 1991; Merino et al. 1993; Yokomori et al. 1993; Auble et al. 1994; Ge and Roeder 1994a; Ge and Roeder 1994b; Shykind et al. 1995; Ma et al. 1996; Orphanides et al. 1996). Thus, TFIIA is dispensable for a correct initiation but is also involved in transcriptional activation functioning as either an antirepressor or a coactivator (Kang et al. 1995; Ma et al. 1996).

5.3. The Mediator

Most of the transcriptional coactivators (TAFs, TFIIA, chromatin modifiers) are not required for expression of all Pol II genes. The most universal cofactor transmitting regulatory information to the transcription machinery, in particular to the RNA Pol II enzyme, is a large modular complex known as Mediator (Myers and Kornberg 2000). The main function of mediator is to mediate the interaction between the transcriptional activator and the transcription machinery, in particular the RNA Pol II. Mediator stimulates both basal transcription and activated transcription.

Furthermore, Mediator stimulates *in vitro* the CTD phosphorylation activity of TFIIH (Kim et al. 1994). In yeast it has been shown that Mediator plays also a role in transcription reinitiation (Yudkovsky et al. 2000).
Mediator complexes have first been identified in yeast, were they have been purified as part of the RNA Pol II holoenzyme (Thompson et al. 1993; Kim et al. 1994; Koleske and Young 1994). Later, mediator complexes have been found in all eukaryotes examined, although the Mediator subunits are the least conserved of all the members of the transcription machinery, consistent with the idea that many mediator subunits serve as regulatory factor targets (Malik and Roeder 2000; Myers and Kornberg 2000; Boube et al. 2002). Despite considerable variation in subunit composition of Mediator complexes from yeast, mouse and man, the overall structure of the complexes is very similar (Asturias et al. 1999; Dotson et al. 2000), and the three characteristic domains are always recognizable: the tail, the middle domain and the head, the last two making contacts with Pol II (Asturias et al. 1999).

5.4. USA factors (upstream stimulatory activity)

The cofactors belonging to the USA (upstream stimulatory activity) class interact with the PIC to repress transcription in the absence of activators or to stimulate transcription in the presence of activators (Meisterernst et al. 1991).
Initially, cofactors belonging to this class were classified in positives (PC) and negative cofactors (NC), although now some factors seem to play a role in both activation and repression of transcription and also new general cofactors have been discovered with the time.
To the group of positive cofactors belong PC1 (later identified as a poly(ADP-ribose) polymerase, PARP (Meisterernst et al. 1997), PC2, a component of the mediator complex (Kretzschmar et al. 1994; Malik et al. 2000); PC3/Dr2, which is the topoisomerase I and functions in both repression of basal transcription and stimulation of activated transcription (Kretzschmar et al. 1993; Merino et al. 1993), PC4, PC5, PC6. To now, the activities of these factors have been identified and differently characterized (Kaiser and Meisterernst 1996). The nonhistone chromosomal protein HMG2 was also identified as a transcriptional activator. This architectural protein functions as a coactivator by stabilizing an activated form of PIC (Shykind et al. 1995). PC1, PC3, PC4, and HMG2 are all nonsequence-specific DNA binding proteins, suggesting that these cofactors function by affecting the accessibility of RNA Pol II to chromatin (Kaiser and Meisterernst 1996).
The most known positive cofactor is PC4. PC4 dramatically stimulates activated

transcription through direct interaction with various activator domains and with the DNA-TBP-TFIIA complex (Ge and Roeder 1994b).

The group of negative cofactors includes NC1 and NC2 (discussed in detail below). NC1 represses basal transcription binding to the TFIID-DNA complex, activity that is competed by binding of TFIIA to the same complex (Meisterernst et al. 1991). Among the general cofactors with negative effect in transcription there are also the HMG1 (high mobility group) and HMG2 proteins and Ada1/Mot1 (Kaiser and Meisterernst 1996).

5.5. BTAF1

Human BTAF1 (formely $TAF_{II}170$/TAF-172) and its yeast ortholog, Mot1p, are general repressors of Pol II-dependent transcription (Davis et al. 1992). BTAF1 and Mot1p possess an ATPase activity and dissociate TBP from TATA-DNA complexes using the energy of ATP hydrolysis (Chicca et al. 1998; Pereira et al. 2003) This activity is not restricted to specific promoters and is counteracted by TFIIA and, partially, also by TFIIB, although the exact mechanism is not fully understood. In mammalian cells the majority of TBP is not found in TFIID but in a complex with BTAF1, called B-TFIID (Timmers and Sharp 1991).
Recent data have revealed BTAF1 and Mot1p as positive regulators of TBP function in the Pol II system. Genome wide transcriptional profiling analysis and mutational studies suggest that Mot1p positively and negatively affects transcription of subsets of yeast genes (Collart 1996; Prelich 1997; Muldrow et al. 1999; Andrau et al. 2002; Dasgupta et al. 2002; Geisberg et al. 2002). Thus, these factors can function either as an activator or as a repressor of transcription. To reconcile the opposite effects on transcription, it has been hypothesized that BTAF1 and Mot1 delocalize TBP from non-promoter regions, in an ATP-dependent manner, and deliver it to promoter sites (Muldrow et al. 1999; Geisberg et al. 2002).

6. Chromatin structure

The compaction of DNA that occurs through packaging is necessary to fit into the limiting confines of the nucleus. DNA is packaged into a nucleoprotein complex known as chromatin.
The fundamental repeating unit of chromatin is the nucleosome, which includes

a histone core (made of two of each of the four histones H2A, H2B, H3, H4), one molecule of H1 or H1-like linker histone, and the associated DNA, usually about 200 bp long, (Kornberg 1977; Chambon 1978). Histones proteins are highly conserved among eukaryotes and consist of a globular domain and a N-terminal unstructured tail. Unlike the four histones H3, H4, H2A, H2B, the lysine-reach histone H1 is not essential for the basic chromatin structure. Also non-histone proteins, like HMG- or SIR-proteins, bind to DNA producing a compact, higher order structure (Naar et al. 2001).

6.1. Histone modifications and histone modifying complexes

Histones serve to pack DNA into compact chromatin structure. However, during specific phases, replication-, transcription-, and repair-factors need to access specific DNA sequences. For this purpose, chromatin organization is regulated by covalent modifications of the histones themselves, association of non-histone proteins, and ATP-dependent mobilization of nucleosomes (Strahl and Allis 2000; Becker and Horz 2002; Berger 2002; Geiman and Robertson 2002; Narlikar et al. 2002). Also substitution of histones with certain histone variants and corresponding modifications of DNA can have an effect on chromatin structure (Hake et al. 2004). Histones undergo several post-translational modifications, like phosphorylation, acetylation and methylation. In many instances, the precise sites of modifications have been identified.
A breakthrough in understanding the role of histone modification came from the discovery that some transcription cofactors are able to modify histones, suggesting a direct link between transcription and histone modifications (Bannister and Kouzarides 1996; Brownell et al. 1996; Ogryzko et al. 1996).

The discovery of histone tails modifications and that these are recognized by different classes of remodeling proteins, led to the so-called histone-code model (Strahl and Allis 2000; Jenuwein and Allis 2001). This model proposes that specific combinations of histone tail modifications provide binding sites for effector proteins, which in turn translate this code into chromatin structural changes. Thus, these modifications may serve as a code to initiate specific cellular actions on the DNA template, such as mitosis, transcription, or replication. For example, acetylation is a mark for the regions of the genome that are competent for transcription, as it is the case for acetylation of H3 and H4 tails (Eberharter and Becker 2002).

6.1.1. Acetylation and HATs/HDACs

Histone acetylation is the best understood of the histone modifications, both in terms of the residues affected and the consequences for transcriptional activity. Several lysines on the N-terminal tail of each of the core histones can be reversibly acetylated.

Histone acetylation neutralizes the positive charges on lysines, weakening the tight interaction with the DNA and neighboring nucleosomes (Allfrey et al. 1964; Luger and Richmond 1998; Berger 2001). Acetylation may also influence transcription by promoting or suppressing interactions with specific transcription factors (Garcia-Ramirez et al. 1995; Dhalluin et al. 1999; Ornaghi et al. 1999).

The enzymes responsible for these modifications are called histone acetyltransferases (HATs) and they all contain a bromodomain, which binds acetylated Lys (Dhalluin et al. 1999).

After initial discovering that the yeast transcription factor Gcn5 has HAT activity, many others HATs have been described, that are now divided in three families (Gregory et al. 2001; Marmorstein and Roth 2001). In addition, also CBP/p300, TAF1, TAFIIIC, and Nut1 posses HAT activity.

Histones are not the only substrate for HATs. Several transcription factors including E2F (Martinez-Balbas et al. 2000) and p53 (Gu and Roeder 1997) have been shown to be acetylated.

Lysine acetylation is reversed by histone deacetylases (HDACs) (Marks et al. 2003). Like HATs, HDACs are part of multiprotein complexes, which have different substrate specificities and corepressor interactions.

6.1.2. Phosphorylation

Histone tails are also modified by phosphorylation on serine or threonine. Phosphorylation of histones H1 and H3 has been implicated in chromosome condensation during mitosis (Bradbury et al. 1974; Bradbury 1992; Berger 2002), although a revised model indicates H3-S10 phosphorylation as a signal for the cells to proceed from metaphase to the anaphase (Hans and Dimitrov 2001).

6.1.3. Methylation and histone methyltransferases (HMTases)

Histone can be methylated both in the lysine and in the arginine residues. Arginines can be mono- or di-methylated by the protein R-methyltransferases (PRMTs), whereas lysines can be mono-, di- or tri-methylated by the HMTs. Besides one exception (Feng et al. 2002), all the Lys methyltransferases contain the so-called SET domain.

Arginine methylation of histones has so far only been associated with transcriptional

activation (Kouzarides 2002), whereas the effect of lysine methylation can have opposite effects (Lachner and Jenuwein 2002).
The first HMTase discovered was the Lysine-methyltransferase SUV39H1 (Rea et al. 2000), followed by many others. Methylation of H3-K9 leads to conversion of chromatin in the transcriptionally inactive heterochromatin (Jenuwein and Allis 2001; Lachner and Jenuwein 2002). Interestimgly, lysine methylation on the same histone tail (H3-K4) results in transcriptional activation (Strahl et al. 1999; Xu et al. 2001; Santos-Rosa et al. 2002).

Lysine methylation is a very stable histone modification and so far no demethylase has been found. However, since lysine methylation is associated with constitutive heterochromatin and transient transcription regulation, the existence of demethylases is plausible. Alternatively, histones could be replaced with unmodified histones during replication or through degradation of the histone tails (Bannister et al. 2002).

6.1.4. Additional histones modifications

Ubiquitination, ADP-ribosylation, biotinylation and SUMOyation have also been reported to be histones modifications.
Histones can be reversibly ubiquitinated, especially on histone H2A but also on H2B and H3 (Chen et al. 1998; Pham and Sauer 2000), and these ubiquitinated histones are associated with transcriptionally active DNA (Nickel et al. 1989). A regulatory role for histone ubiquitination in transcription is not yet firmly established.
ADP-ribosylation has been connected to transcription, apoptosis and genomic stability (Rouleau et al. 2004). Histones have also been reported to be biotinylated (Camporeale et al. 2004) and SUMOylated (Shiio and Eisenman 2003).

6.2. Non covalent chromatin modification: ATP-dependent Chromatin remodeling complexes

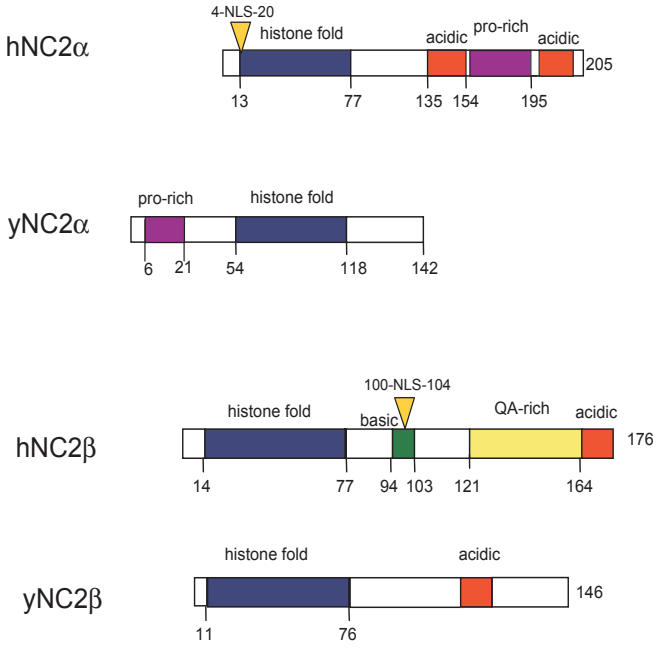
In addition to covalent modifications, nucleosomes are subjected to conformational remodeling (Becker and Horz 2002). Remodeling involves the breaking and reforming of histone-DNA contacts that result in the mobilization of nucleosomes in the chromatin template (Peterson 2002). Although the precise mechanism is unknown, several different remodeling complexes have been identified. All of these complexes contain an ATPase subunit along with additional subunits that affect regulation, efficiency, and specificity.

As for HMTase, also ATPase enzymes can have a double role in transcription repression and activation (Stokes et al. 1996; Tong et al. 1998; Xue et al. 1998).
Chromatin remodeling complexes are divided in 3 major groups, depending on the ATPase activity: SWI/SNF, ISWI and the Mi-2/CHD-Group (Becker and Horz 2002; Peterson 2002; Tsukiyama 2002).

7. The negative cofactor 2, NC2

NC2 was initially purified from human cell extracts as an activity that inhibits transcription initiation by RNA Pol II (Meisterernst and Roeder 1991; Inostroza et al. 1992). NC2 is a heterodimer consisting of two subunits, NC2α and NC2β, also called DRAP1 and DR1, respectively (Goppelt et al. 1996; Mermelstein et al. 1996). Both NC2 subunits have a histone fold domain, necessary for their interaction (Fig. 2). These domains are similar to those found in the histone pair H2A-H2B, which are closely related to those found also in the transcription factors NF-YB/NF-YC, in the yeast HAP3/HAP5 and in the chromatin remodeling proteins CHRAC-15/17 (Corona et al. 2000; Poot et al. 2000). Both subunits are highly conserved in eukaryotes, especially in their histone fold domains (Fig. 3).
The homologous yeast complex is called yNC2α/Bur6p and NC2β/Ydr1p. Both subunits are essential for yeast viability and are functionally interchangeable between yeast and human (Goppelt and Meisterernst 1996; Gadbois et al. 1997; Kim et al. 1997; Prelich 1997; Lemaire et al. 2000; Xie et al. 2000). The genes encoding the subunits were identified by sequence analysis of the yeast genome (Goppelt and Meisterernst 1996; Gadbois et al. 1997; Kim et al. 1997) and in two genetic selections for general repressors of transcription. First, *bur6* mutations were identified by their ability to increase transcription from enhancerless promoters, suggesting that NC2 inhibits basal transcription *in vivo* (UAS-less *SUC2* promoter) (Prelich and Winston 1993; Prelich 1997), both bur6 and ydr1 mutations were found to suppress mutation in SRB4, which encodes a subunit of the Mediator complex (Gadbois et al. 1997; Lee et al. 1998). Expression of high copy number of *YDR1* diminished mRNA accumulation and caused slow growth of yeast, consistent with its role of general transcriptional repressor. Moreover, the slow growth phenotype is partially suppressed by overexpression of TBP (Kim et al. 1997).

A



B

hNC2α

```
MPSKKKKYNA  RFPPARIKKI  MQTDEEIGKV  AAAVPVIISR  ALELFLESLL  50
KKACQVTQSR  NAKTMTTSHL  KQCIELEQQF  DFLKDLVASV  PDMQGDGEDN  100
HMDGDKGARR  GRKPGSGGRK  NGGMGTKSKD  KKLSGTDSEQ  EDESEDTDTD  150
GEEETSQPPP  QASHPSAHFQ  SPPTPFLPFA  STLPLPPAPP  GPSAPDEEDE  200
EDYDS                                                     205
```

hNC2β

```
MASSSGNDDD  LTIPRAAINK  MIKETLPNVR  VANDARELVV  NCCTEFIHLI  50
SSEANEICNK  SEKKTISPEH  VIQALESLGF  GSYISEVKEV  LQECKTVALK  100
RRKASSSRLEN  LGIPEEELLR  QQQELFAKAR  QQQAELAQQE  WLQMQQAAQQ  150
AQLAAASASA  SNQAGSSSQDE  EDDDDI                  176
```

Figure 2. (A) Schematic drawing of NC2α and NC2β from human (h) and S. cerevisiae (y). The histone fold domain, the acidic and the basic residues are indicated with different colours. The potential nuclear localization signals (NLS) (numbers refer to first and last residues in the sequence) are represented by triangles. **(B)** Amino acid sequence of the two NC2 subunits. hNC2α has 205 amino acids and a predicetd molecular weight of 22.3 kD. hNC2β has 176 amino acids and weights 19.4 kD. In blue are the NLS studied in the present work. Potential phosphorylation sites are in bold. Underlined residues are recognition sites for the kinase CKII, the red ones for the kinase PKA and the green for both the kinase PKA and PKC.

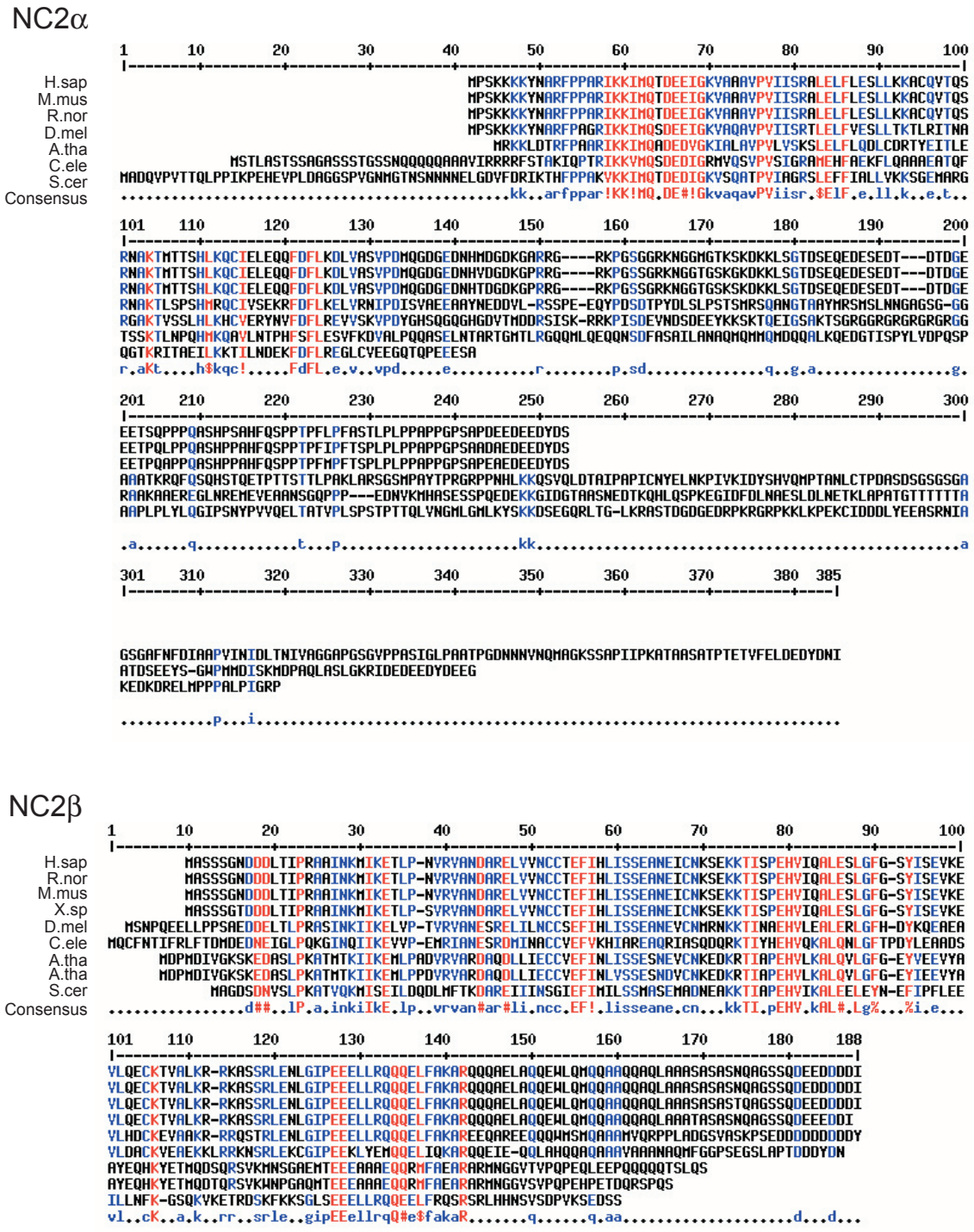


Figure 3. Alignement of NC2α (upper panel) and NC2β (bottom panel) from different species. H.sap = *Homo sapiens*; M.mus = *Mus musculus*; R.nor = *Rattus norvegicus*; D.mel = *Drosophila melanogaster*; X.sp. = Xenopus spp.; S.cer = Saccaromices cerevisiae; A.tha = *Arabidopsis thaliana*.

7.1. NC2 is a general transcriptional repressor

In vitro, NC2 associates with promoter-bound TBP, thereby preventing the recruitment of TFIIA and TFIIB to the promoter (Meisterernst and Roeder 1991; Inostroza et al. 1992; Kim et al. 1995; Goppelt et al. 1996; Mermelstein et al. 1996).

NC2 globally represses Pol II and Pol III mediated transcription, but not Pol I transcription (White et al. 1994).

The crystal structure of NC2 in a complex with TBP-DNA provided a model for the repression mechanism of NC2 (Fig. 4). This structure shows that the N-terminal ends of NC2α and NC2β, containing the histone fold domain, form an intimate heterodimer that binds to the under side of the TBP-DNA complex. The C-terminus of NC2β makes specific contacts with the convex surface of the TBP saddle and blocks entry of TFIIB into the transcription complex (Kamada et al. 2001). This is consistent with the previous observations that TBP mutations that prevent the

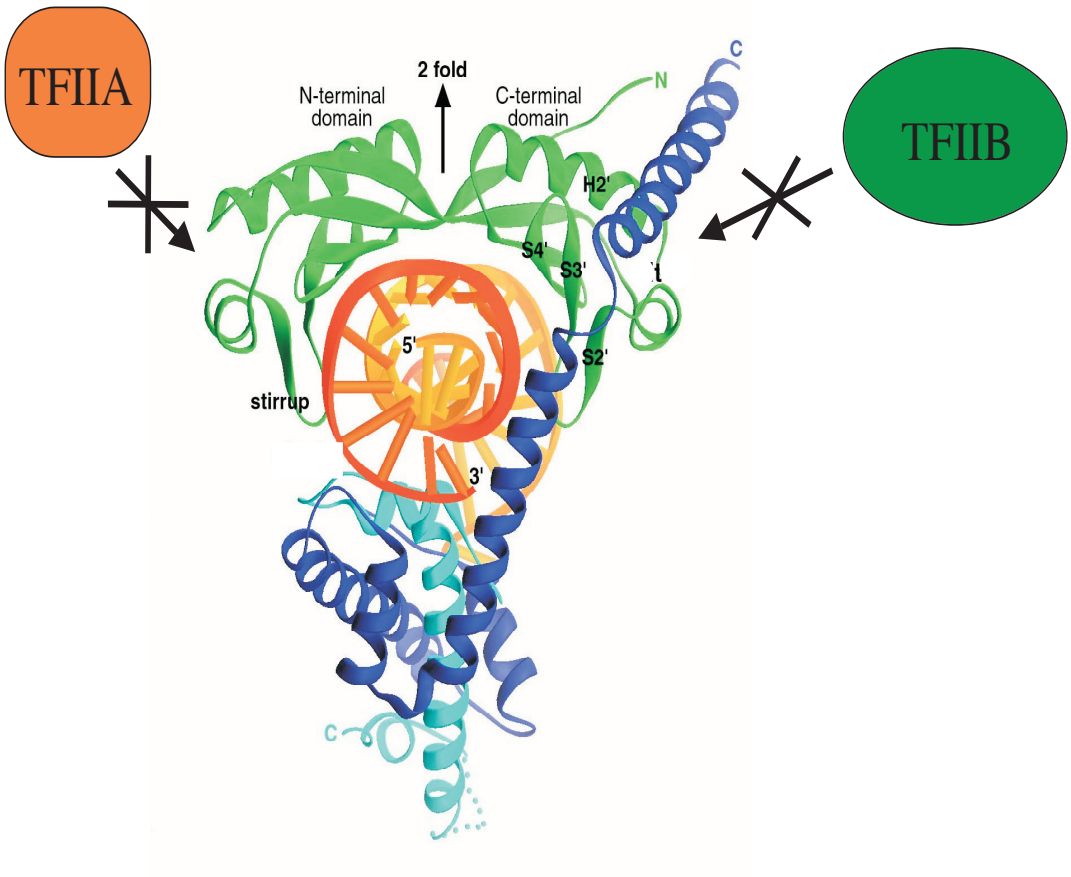


Figure 4. Schematic representation of the crystal structure of the NC2-TBP-DNA complex. In orange is the DNA; TBP is in green and NC2 in blue (NC2α in light, NC2β in dark). NC2 binding to TBP-DNA prevents recruitment of TFIIA and TFIIB in the promoter (after Kamada et al. 2001).

interaction with NC2 locate near the surfaces of TBP that also mediate association with TFIIB (Cang et al. 1999).
It is not yet clear how NC2 competes with TFIIA for TBP binding, since the two proteins have distinct interaction domains with TBP (Maldonado et al. 1999). Comparison of NC2/TBP/DNA and TFIIA/TBP/DNA structures suggests that TFIIA and NC2 could bind to TBP simultaneously, albeit with lower affinity than for either molecule alone (Bleichenbacher et al. 2003). However the superposition of the structures does not take in account that NC2-TBP-DNA structure does not contain the C-terminus of NC2α, which could also make additional contacts with TBP (Gilfillan et al., submitted; Klejman et al. 2004). NC2 efficiently displaces TFIIA on TBP-promoter complexes and this biochemical data is supported by genetic interaction studies in yeast (Xie et al. 2000).
In support of the negative function is also the finding that in conditions of hypoxia, mammalian cells can induce selective transcription repression increasing NC2 activity (Denko et al. 2003).

7.2. Interplay between negative and positive effectors

Increasing evidence suggest that NC2 is functionally related to the Pol II holoenzyme. Genetic studies established a functional interaction between NC2 and distinct subunits of the Mediator component of the yeast Pol II holoenzyme. (Gadbois et al. 1997; Lee et al. 1998; Kim et al. 2000; Lemaire et al. 2000). The Srb4 protein is an essential component of the RNA polymerase II holoenzyme. Suppressors of Srb4 mutation included partial loss-of-function mutations in the negative regulators NC2 and the Not complex (Lee et al. 1998). Moreover, defects in either subunits of NC2 can be rescued by loss-of-function mutations in the Sin4 subunit of the mediator (Gadbois et al. 1997; Kim et al. 2000; Lemaire et al. 2000). Thus, defects in positive regulators of transcription can compensate for loss-of-function mutations in negative regulators of transcription and vice versa. This observation strengthens the argument for an active interplay between positive and negative regulators *in vivo.*

7.3. Positive role of NC2 in transcriptional regulation

In addition to its well-characterized role as a repressor, several experiments suggest that NC2 might also play a positive role in transcription. In yeast, *bur6* mutations reduce transcription from many promoters (Prelich 1997; Geisberg et al. 2001) and

a slow-growth NC2β mutant inhibited transcription from TATA-containing promoters but stimulated TATA-less-promoters of the *HIS3* and *HIS4* genes (Lemaire et al. 2000). In *Drosophila*, NC2 stimulate *in vitro* transcription from promoters containing downstream promoters elements (DPE), whereas it represses transcription from TATA-containing promoters (Willy et al. 2000). Recently, Cang and Prelich provided the first evidence that yeast NC2 directly stimulates activated transcription in yeast from TATA promoters (Cang and Prelich 2002). Also, gene occupancy experiments in yeast correlated the presence of NC2 on promoters with gene activation (Geisberg et al. 2001). NC2 was also found to be associated with a hyperphosphorylated form of Pol II, but the meaning of this interaction is still unknown (Castano et al. 2000).

8. Post-translational protein modifications: the role of phosphorylation in the regulation of transcription factors

During the past decade, there have been major advances in understanding how post-translational modifications of transcription factors can modulate gene expression changes. The most common mechanism to regulate (positively and negatively) the activity of many eukaryotic transcription factors is certainly protein phosphorylation (Hunter and Karin 1992; Jackson 1992; Whitmarsh and Davis 2000; Holmberg et al. 2002). Although the importance of other types of covalent modifications, such as acetylation, ubiquitination and sumoylation, is now emerging, phosphorylation has several features that make it ideal for regulating transcription factor activity. First, phosphorylation can be very rapid. For example, changes in c-Jun phosphorylation upon phorbol esters stimulation occur within 15 min (Boyle et al. 1991). Second, it is readily reverted by phosphatases, permitting transcriptional regulation to operate in a highly dynamic way. Third, phosphorylation is very effective at integrating information from a number of signal transduction pathways. Fourth, in some cases a single kinase can have different effects on different transcription factors. Finally, phosphorylation can affect different aspects of transcription factor function and regulation.

Serine, threonine, and tyrosine are the amino acids that can be phosphorylated. Addition of a negative charge by phosphorylation can induce allosteric conformation changes, as well as repulsive and attracting forces, thus affecting protein-protein or protein-DNA interactions (Sprang et al. 1988; Hurley et al. 1990).

Protein phosphorylation and dephosphorylation can regulate transcription factor function by at least five different mechanisms (Fig. 5).

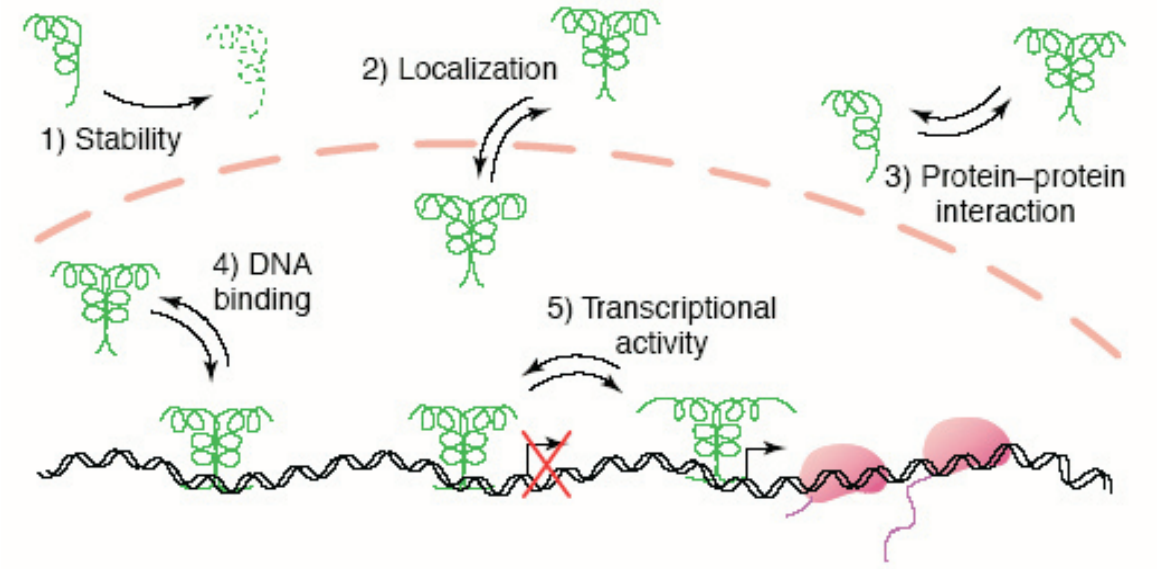


Figure 5. Regulation of transcription factors by phosphorylation. Five different mechanisms of regulation are represented. The transcription factor is depicetd in green, RNA Pol II in red, and the mRNA in black (after Holmberg et al. 2002).

(1) Phosphorylation can affect the stability of the transcription factor in both directions, protecting protein from degradation (*e.g.* ATF2 and p53; Appella and Anderson 2000; Fuchs et al. 2000) as well as promoting proteolysis (*e.g.* MyoD and EsF-1; Song et al. 1998; Vandel and Kouzarides 1999; IkB inhibitory subunit of NFkB, Yaron et al. 1998; Spencer et al. 1999). In the second case, protein phosphorylation allows recognition of the transcription factor by a group of enzymes (E1, E2, and E3) that covalently attach ubiquitin to the target protein. The ubiquitinated proteins are than degraded by the 26S proteosome (Hershko and Ciechanover 1998).

(2) Phosphorylation can regulate the subcellular localization of a protein. Many transcription factors are constitutively nuclear, and are phosphorylated and dephosphorylated by protein kinases and protein phosphatases within the nucleus. However, a number of transcription factors shuttle between the cytoplasm and the nucleus, and in many cases this process is regulated by protein phosphorylation/dephosphorylation. The nucleocytoplasmic shuttling of transcription factors is an active process that relies on the recognition of nuclear localization signals (NLS) and nuclear export signals (NESs), present in the transcription factor primary sequence, by proteins of the nuclear import and export machinery (Hood and Silver 1999). Protein phosphorylation and dephosphorylation can however regulate the accessibility of the NLS and NESs to the nuclear import and export proteins in at least two ways: (i) by directly masking or unmasking the transcription factor signal, or (ii) by modulating the binding of the transcription factor to other regulatory

proteins which mask or unmask the signal sequences.
In the first case, phosphorylation usually prevents nuclear import, while dephosphorylation activates it. For example, NFAT and FKHRL1, a member of the Forkhead family, are both retained in the cytoplasm when highly phosphorylated (Zhu et al. 1998; Biggs et al. 1999; Brunet et al. 1999; Brunet et al. 2001b).
A good exemple of the second mechanism is the heterodimeric transcription factor nuclear factor-κB (NF-κB). NF-κB is regulated by binding to the cytoplasmic anchoring protein inhibitor of NF-κB (I-κB) (Ghosh et al. 1998). The binding of I-κB to NF-κB masks the NLS located on the p65 subunit of the NF-κB heterodimer, thereby preventing translocation of NF-κB to the nucleus (Ghosh et al. 1998). Phosphorylation of I-κB targets this subunit for degradation, allowing exposure of the NLS on p65 and transport to the nucleus.

(3) Phosphorylation can modulate protein-protein interactions. This can occur by (i) blocking of an interaction surface following phosphorylation; (ii) by a phosphorylation-induced conformational change that unmasks or masks the binding surface; (iii) by phosphorylation causing the dissociation of an inhibitor molecule to unmask a binding surface.
A well-known example of how protein phosphorylation can regulate the interaction of transcription factors with cofactors is represented by the transcription factor cAMP-response element binding protein (CREB). When phosphorylated at a certain residue, CREB binds to the coactivator protein CBP, which links CREB with components of the basal transcription machinery, leading to increased transactivation (De Cesare et al. 1999).

(4) Phosphorylation affects the DNA-binding activity. Many DNA binding domains are basic in character, so phosphorylation within or nearby these domains introduces a negative charge, which may be incompatible for efficient DNA binding. For example, the DNA binding activity of c-Jun is both positively and negatively regulated by phosphorylation in a site-specific manner (Whitmarsh and Davis 2000).

(5) Phosphorylation regulates the transcriptional activity of a factor such as HSF1, the mediator of stress-induced expression of heat shock genes (Knauf et al. 1996; Holmberg et al. 2002). In response to heat shock and other protein-damaging stresses, HSF1 undergoes nuclear localization, binds to heat shock elements, becomes phosphorylated and subsequently acquires transcriptional activity (Pirkkala et al. 2001).
In addition, phosphorylation can also regulate transcription by modifying chromatin

structure and the accessibility of promoter binding sites to transcription factors. These changes are mediated by multisubunit ATP-dependent remodeling complexes or by covalent modification of the nucleosomes (Workman and Kingston 1998).
All these regulatory processes are fully integrated, and the outcome depends on the types of signals operating at a particular time and place in the cell.

9. Silencing of transcription during mitosis

The typical eukaryotic cell cycle comprises 4 phases (Fig. 6). During mitosis (the M phase) the cell divides into two daughters cells. The M phase comprises mitosis and cytokinesis (Earnshaw and Pluta 1994; King et al. 1994). With mitosis, sister chromatids segregate into two nascent cells, such that each daughter cell inherits one complete set of chromosomes. In addition, each daughter cell must receive one centrosome and the appropriate complements of cytoplasm and organelles.
After the M phase, the two daughters cells enter in the interphase of a new cycle. The interphase begins with the G1 phase, during which the biosynthetic activity of the cell starts again at high speed.
The S phase is so called because the synthesis of the DNA takes place, and this phase ends only when chromosome duplication is accomplished. The S phase is followed by a second gap phase, called G2, which precedes the entry in a new cell division.

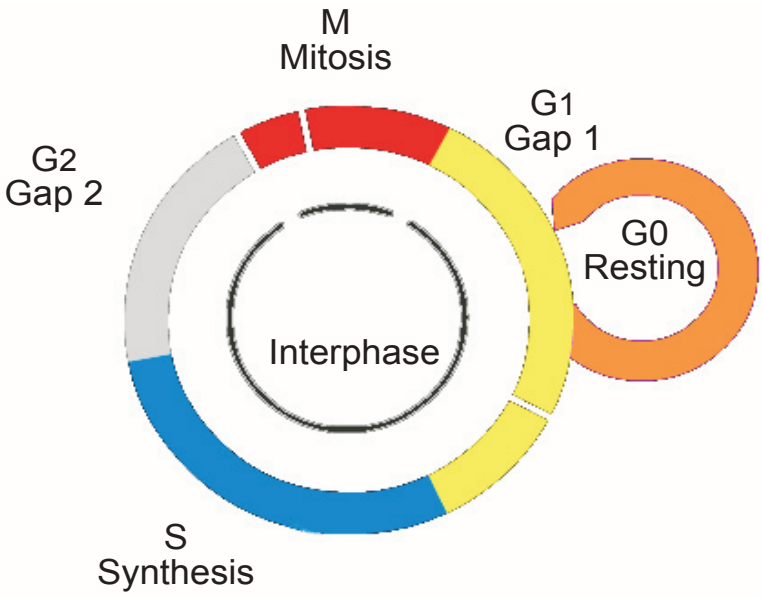


Figure 6. Schematic representation of the cell cycle phases.

The regulation of the M phase progression relies predominantly on two mechanisms: protein phosphorylation and proteolysis. These are intimately intertwined, in that the proteolytic machinery is controlled by phosphorylation, whereas several mitotic kinases are downregulated by degradation.
The most prominent mitotic kinase is the founding member of the Cdk family of cell-cycle regulators, the cyclin-dependent kinase1, Cdk1, also called cdc2 (Coleman and Dunphy 1994; Elledge and Harper 1994; King et al. 1994). The activity of the Cdks is modulated through phosphorylation of their catalytic subunits, and by their association with positive (cyclins) and negative regulatory proteins (Elledge and Harper 1994). Distinct cyclins perform different tasks in specific phases of the cell cycle. The cyclin associated to the cdc2 kinase is the cyclin B. The complex formed by the kinase and the cyclin, cdc2/cyclinB, is also called MPF, for mitosis promoting factor (Labbe et al. 1988; Gautier et al. 1990). Activation of MPF results in the activation of different kinases and the inhibition of the phosphatases PP1 and PP2A (Nigg 1993). As a result, during mitosis multiple proteins are phosphorylated, causing a reorganization of the nuclear envelope, the spindle apparatus, the chromosomes, and the regulation of transcription factors.
Recent studies brought to light additional mitotic kinases. These include members of the Polo family, the Aurora family and the NIMA (never mitosis A) family, as well as kinases implicated in mitotic checkpoints, mitotic exit and cytokinesis (Nigg 2001).

When cells enter mitosis, transcription of the eukaryotic genome, which is highly active in interphase, is abruptly silenced. Experiments performed already 40 years ago showed that *in vivo* incorporation of radioactive precursors into nuclear RNA declines in early to mid-prophase and resumes in late telophase (Prescott and Bender 1962; Konrad 1963; Terasima and Tolmach 1963). The precise degree of mitotic transcription repression remains uncertain, with some studies detecting mitotic RNA synthesis at 16 to 24% of the interphase levels (Konrad 1963; King and Barnhisel 1967). Indeed, mitotic repression of transcription has been observed *in vivo* for genes transcribed by all three nuclear RNA polymerases (Prescott and Bender 1962; Fink and Turnock 1977). Approximately 75 to 80% of RNA synthesis in cycling cells is due to RNA Pol I activity (Reeder and Roeder 1972; Love and Minton 1985; Zawel and Reinberg 1995), but it is not clear to what degree transcription by each of the three nuclear polymerases is repressed during mitosis.
Mitotic repression has been associated to a number of regulatory mechanisms (Hartl et al. 1993; Martinez-Balbas et al. 1995; Gottesfeld and Forbes 1997), including

condensation of interphase chromatin into mitotic chromosomes (Fink and Turnock 1977), dissociation of transcription factors or RNA polymerases from the chromatin template (Martinez-Balbas et al. 1995; Parsons and Spencer 1997), and premature termination of transcription observed for very large genes in *Drosophila* (Shermoen and O'Farrell 1991).
Chromatin condensation naturally limits the accessibility of the DNA template to transcription factors and RNA polymerase, and is mediated by the DNA topoisomerase II (Hirano and Mitchison 1991) and the phosphorylation of the linker histone H1 and the core histone H3 (Bradbury et al. 1974). Moreover, genetic and biochemical studies have uncovered a multisubunit complex, named condensin, required for mitotic chromosome structure and condensation (Hirano and Mitchison 1994; Swedlow and Hirano 2003; Watrin and Legagneux 2003).

Besides chromatin condensation, a key role in mitotic inactivation of transcription is played by phosphorylation of basal transcription factors and of RNA Pol II (Gottesfeld et al. 1994; Leresche et al. 1996; Segil et al. 1996; Akoulitchev and Reinberg 1998; Long et al. 1998).
In fact, mitotic repression of transcription by RNA Pol II can be reproduced *in vitro* with asynchronous cell extracts treated with the master mitotic kinase cdc2/cyclin B. This effect is reversed by specific inhibition of this kinase, indicating that repression of transcription is due to protein phosphorylation (Gottesfeld et al. 1994; Martinez-Balbas et al. 1995).
Potential targets for this repression are the general transcription factors, the RNA Pol II itself and also activator proteins that bind enhancer or upstream elements.
Earlier studies showed that both TBP and some TAF components of the TFIID complex are phosphorylated in mitosis (Segil et al. 1996). This modification selectively inhibits activator-dependent transcription, but not activator-independent basal transcription *in vitro* (Segil et al. 1996), indicating that the mitotic phosphorylation of TFIID subunits does not affect TFIID promoter binding activity itself. Also TFIIH is inactivated in mitosis (Akoulitchev and Reinberg 1998; Long et al. 1998). The Cdk7 subunit of the CAK complex of TFIIH is phosphorylated by cdc2/cyclin B and this modification impairs CTD-kinase activity and therefore transcription activities of TFIIH (Akoulitchev and Reinberg 1998).
Thus, for an activated promoter, targets of repression are TFIID and TFIIH, while for a basal promoter, TFIIH is the major target for mitotic inactivation of transcription.
The CTD domain of RNA Pol II is also phosphorylated by cdc2/cyclinB, and this results in the inhibition of transcription in a reconstituted transcription system containing the basal transcription factors and polymerase (Cisek and Corden

1989; Zhang and Corden 1991; Leresche et al. 1996; Patturajan et al. 1998). CTD phosphorylation may also affect other protein-protein interactions within the initiation complex. Thus, mitotic phosphorylation of the CTD may be an additional mechanism for general repression of Pol II transcription.

More recently, chromatin immunoprecipitation assays showed that the general transcription factors TFIID and TFIIB could remain associated with active gene promoters during mitosis, while RNA Pol II is displaced from the condensed mitotic chromosomes (Christova and Oelgeschlager 2002). Furthermore, the dynamics/ distribution of TBP in living human cells showed that a small fraction of TBP-TAFs complexes stably associate with the condensed chromosomes during mitosis (Chen et al. 2002).

These results prompted the idea that the presence of promoter bound factors could mark genes for rapid transcriptional activation as cells exit from mitosis (Michelotti et al. 1997; Chen et al. 2002; Christova and Oelgeschlager 2002).

During mitosis, not only the basal Pol II machinery is altered, but also specific Pol II transcription factors have been reported to be phosphorylated. For example, Oct-1, a transcription factor used by a subset of genes transcribed by Pol II and Pol III, is hyperphosphorylated as cells enter mitosis and dephosphorylated as cells exit mitosis (Segil et al. 1996). Phosphorylation of Oct-1 negatively regulates its DNA binding activity (Segil et al. 1991). Another member of the POU family of homeodomain proteins, GHF-1, is also subject to mitotic phosphorylation, and this event inhibits the DNA-binding activity of GHF-1 (Caelles et al. 1995). The general Pol II factor Sp1 and the oncoproteins Myb and Myc are also hyperphosphorylated at mitosis and exhibit reduced binding activity in mitotic cell extracts (Luscher and Eisenman 1992; Martinez-Balbas et al. 1995). In addition to DNA-binding transcription factors, also two components of the human SWI-SNF complex, involved in transcriptional activation through opening chromatin structure, are phosphorylated at mitosis and are excluded from mitotic chromosomes, contributing to mitotic repression (Sidorova et al. 1995; Muchardt et al. 1996).

Thus, phosphorylation of transcription factors seems to play a major and widespread role in regulating transcription during mitosis.

10. Protein transport to the nucleus

10.1. The nuclear pore complex (NPC)

In the cells of eukaryotic organisms, the nucleus is separated from the cytoplasm by a nuclear envelope: this consists of two bilayer membranes (the inner nuclear membrane and the outer nuclear membrane), the nuclear pore complexes (NPCs) and the nuclear lamina.
The NPCs are embedded in the nuclear envelope and provide spatial connection between nucleus and cytoplasm. The pore complexes are large proteinaceous structures with an estimated molecular mass of 60 MDa in *S. cerevisiae* and 125 MDa in *vertebrates*, ca 40 times bigger than a ribosome (Reichelt et al. 1990; Rout and Blobel 1993). The number of NPCs present in the nuclear envelope is usually proportional to the metabolic activity of the cell.
NPCs have a highly conserved architecture with an octagonal symmetry. Elements characteristic of this structure include spokes, rings, a central channel, cytoplasmic fibrils and a nuclear basket (Garcia-Bustos et al. 1991; Forbes 1992). More than 100 types of proteins, termed nucleoporines, compose the pore complexe (Davis 1995).
In correspondence to the NPCs, the inner and outer membranes are fused, so that the pore complexes create an aqueous channel through which proteins and RNA are transported. Molecules with a molecular mass below 40-50 kDa can pass through the pore by passive diffusion. Transport of bigger molecules requires energy and is mediated by signals present in the cargo molecule. The largest known substrates for active transport are roughly 25–50 MDa. The diameter of the NPC channel can reach a maximum of approximately 25 nm, but the diffusion channel is 9 nm, indicating that the NPC recognizes and reacts to specific transport substrates by undergoing a considerable conformational change.

Active transport of proteins into the nucleus is a rapid, specific and evolutionary conserved process. Studies made with *in vitro* transport systems suggested that transport is accomplished in two stages: (i) binding of the substrate to the cytoplasmic surface of the NPC and (ii) transport through the pore to the other side of the nuclear envelope (Fig. 7). To this purpose, the substrate needs a nuclear localization signal (NLS) to be recognized by the nuclear pore receptors. This process is energy-independent, contrary to the translocation through the pore, which is energy-dependent (Schlenstedt 1996). Proteins containing an NLS are recognized and carried to the pore by the □-importin family of nuclear transport

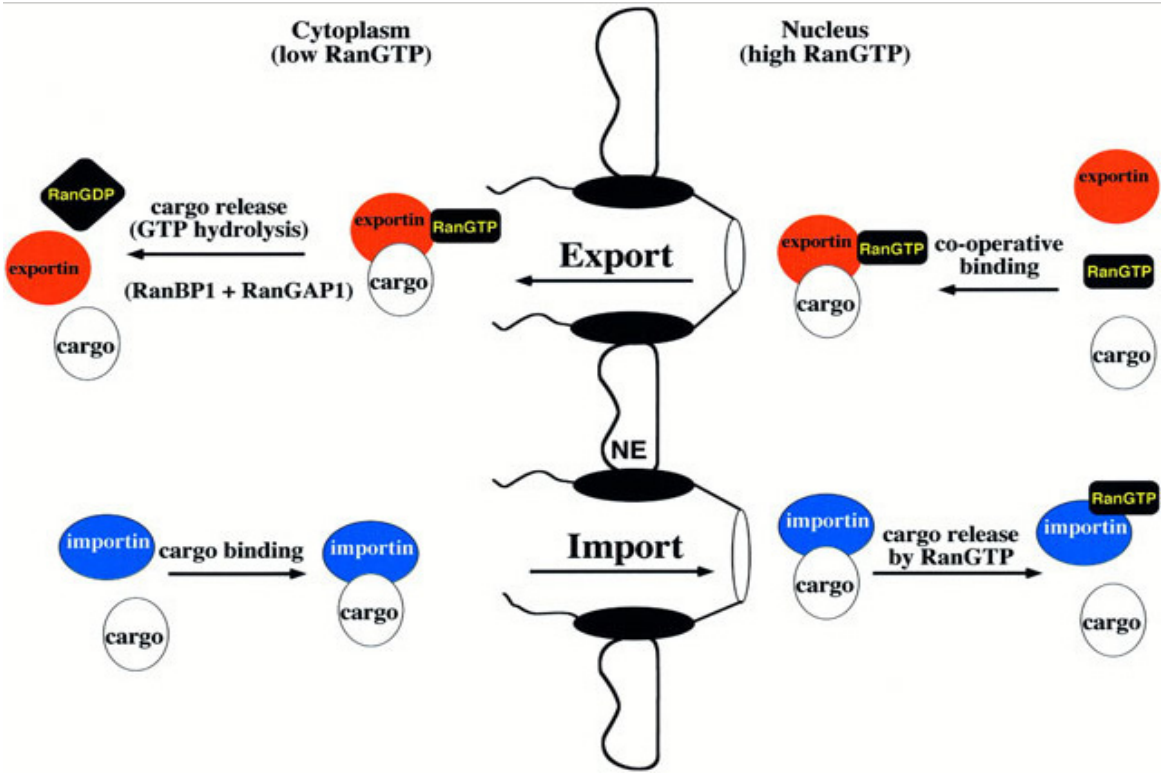


Figure 7. Generalised model of function for import factors (importins) and export factors (exportins). For details see main text. (after Gorlich 1998).

receptors (Ohno et al. 1998), also known as karyopherins, kaps or NLS receptors (NR). The importins direct the nuclear import and export of the cargo in association with the small GTPase Ran. Although Ran is found in both the nucleus and the cytoplasm, its effectors are asymmetrically distributed within the cell. In fact, The Ran GTPase activating protein (RanGAP) is localized to the cytoplasm and the Ran GTP exchange factor (RanGEF) is localized to the nucleus. As a result, nuclear Ran is in the GTP-bound stage, whereas cytoplasmic Ran is GDP-bound, thus ensuring the unidirectionality of the transport (Gorlich and Laskey 1995; Gorlich et al. 1995; Moore 1998).

10.2. The nuclear localization signal (NLS)

The classical NLS can be of two types: (i) short sequences of 4-8 amino acids positively charged, like the prototype NLS from the SV40 large antigen T (Kalderon et al. 1984a; Kalderon et al. 1984b; Lanford and Butel 1984); (ii) bipartite sequences, consisting of two clusters of basic residues separated by a spacing region of ca 10 amino acids (like the bipartite signal of the nucleoplasmin) (Makkerh et al. 1996).

Comparison of different NLS show that there are some general requirements for their functionality, rather than a unique consensus sequence (Garcia-Bustos et al. 1991). NLS are usually short sequences of not more than 8-10 amino acids, with a high proportion of positively charged amino acids (lysines and arginines) and often they contain prolines. A protein can have more than one NLS, with no preferential localization and they are not eliminated after entering into the nucleus. Hence, as soon as the nuclear membrane is reassembled after being dissolved during mitosis, nuclear proteins can rapidly shuttle back to the nucleoplasm.
Most of the imported proteins studied so far contain at least two signals. When more NLS are presents in the protein, there could be one strong signal associated to a secondary weaker signal, or many weak signals that act cooperatively in promoting nuclear import. Yet, it is still difficult to predict the strength based on their sequence.

Studies on the intracellular transport of the heterogeneous ribonucleoproteins (hnRNP) family, and in particular on hnRNP A1, revealed a new type of nuclear targeting signal called M9, composed of a domain of 39 amino acids (Pollard et al. 1996). M9 does not contain basic residues, whereas is rich in aromatic residues and glycines; as expected from the different nature of the characteristic amino acids, the nuclear receptor recognizing M9 is not the importin. Instead, this receptor is called transportin and is a protein of 90 kDa with 24% homology with Importinβ. M9 is active also as nuclear export signal. Thus, in contrast with the classical NLS, M9 drives the bidirectional transport through the nuclear pores. The discovery of a new type of signal, responsible for a way of import independent from the classical one, demonstrates the existence of at least two receptor mediated import ways.

II. RESULTS

1. Localization studies of NC2

NC2 accomplishes its function as a transcription cofactor in the nucleus. None of the previous studies reports the subcellular distribution of NC2α and NC2β. To investigate this aspect, indirect immunofluorescence (IF) experiments were performed in HeLa adherent cells.

1.1. Subcellular distribution of NC2

1.1.1. Localization of NC2α

Exponentially growing cells were fixed, incubated with the antibody (ab.) against this protein (rat 4G7 monoclonal antibody, Mab) and analyzed by confocal microscopy. Immunofluorescence staining shows that NC2α is confined to the nucleoplasm (Fig. 8a). A closer look shows that in some cells the staining is also associated to the nucleoli. Since the signal of the endogenous protein was weak, HeLa cells were also transfected with vectors containing NC2α alone or fused to the His- or flag tags (Fig. 8b). The overexpressed protein was detected with the NC2α monoclonal antibody (Mab) and the high intensity of the signal allowed clear distinction of the overexpressed protein from the endogenous one. Flag-NC2α was detected also with a flag specific ab (M2), which showed exactly the same distribution. The overexpressed NC2α localizes in the nuclei and, surprisingly, strongly accumulates in the nucleoli. The flag-NC2α construct shows also additional distribution patterns, with a strong signal observed around the inner part of the nuclear membrane and also in the cytoplasm. Since this construct is the most highly expressed, probably due to a consensus *Kozak* sequence at the start site, the different distribution is most likely an artefact. Transfection experiments using 293 cells yielded equivalent results.

To confirm that the big nuclear spots observed in transfected cells correspond to the nucleoli, colocalization studies using NC2α and a nucleolar specific protein

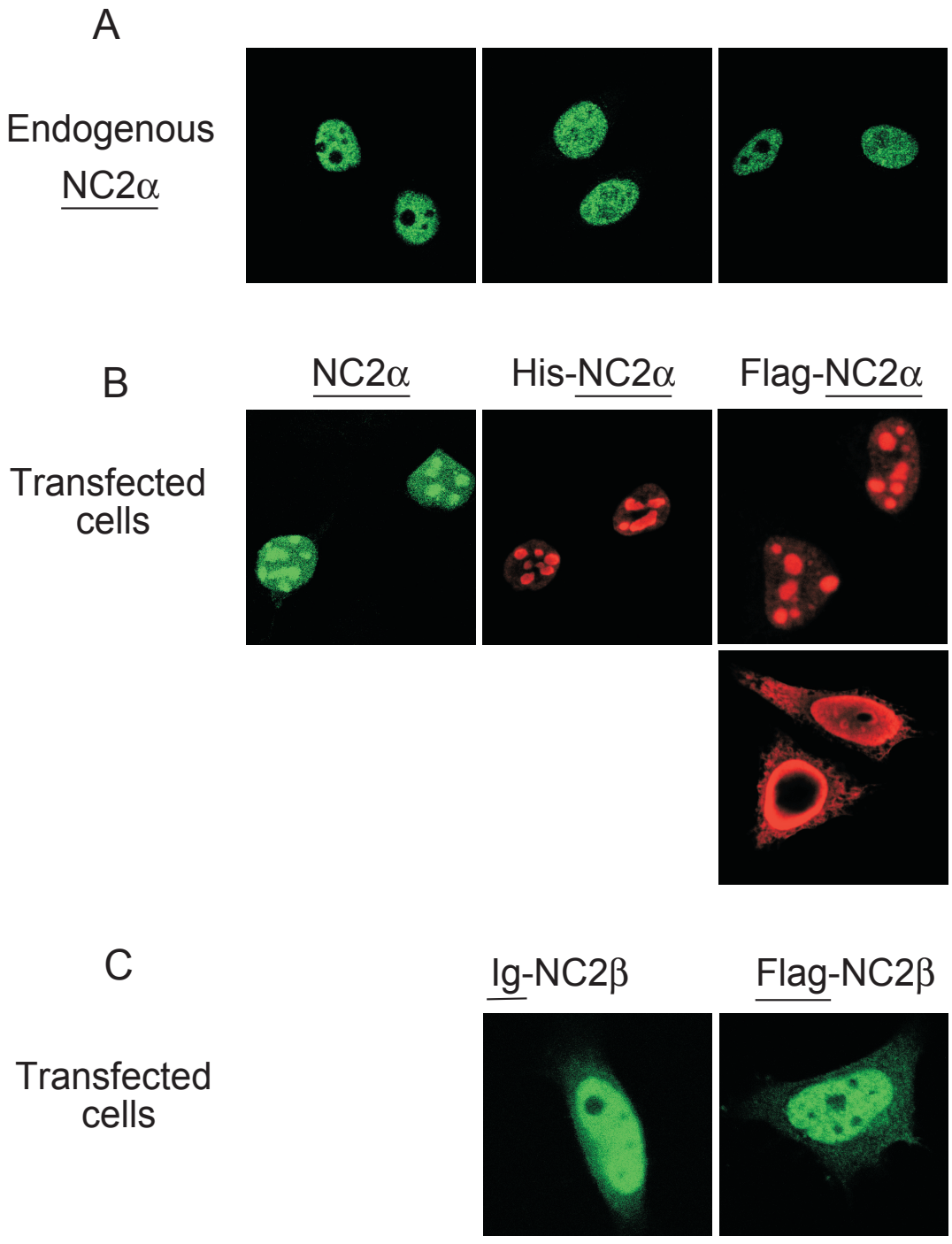


Figure 8. Localization of NC2. (A) Immunofluorescence analysis of NC2α in HeLa cells. Endogenous NC2α is visualized by secondary ab to rat IgG conjugated to biotin and streptavidin Alexa-488-conjugated (green). NC2α localizes in the nucleoplasm and, in some cells, is found also in the nucleoli. (B) HeLa cells were transfected with three different constructs: NC2α, His-NC2α and Flag-NC2α. Cell transfected with NC2α were stained as in A, whereas His-NC2α and Flag-NC2α constructs were incubated with antibody against NC2α and secondary antibody conjugated to the fluorescent dye Cy3 (red). Over expression of NC2α leads to accumulation of the protein in the nucleoli. (C) HeLa cells were transfected with the Ig-NC2β construct and visualized directly with a FITC-conjugated ab directed against the Ig (green). The Flag-NC2β was visualized with an ab against the flag and a secondary FITC anti-mouse (green). NC2β accumulates mostly in the nucleoplasm, but a small fraction is found in the cytoplasm.

(NO38) were performed. This experiment clearly shows that NC2α and NO38 immunostainings colocalize in the nucleoli (Fig. 9a), confirming that NC2α indeed accumulates in the nucleoli in transfected cells.

1.1.2. The nucleolar localization of the endogenous NC2α is restricted to the G1 phase

Since cells were taken from an asynchronous population, we asked whether the ones showing the nucleolar staining corresponded to a specific stage of the cell cycle. During the M phase of the cell cycle nucleoli are disassembled, therefore we

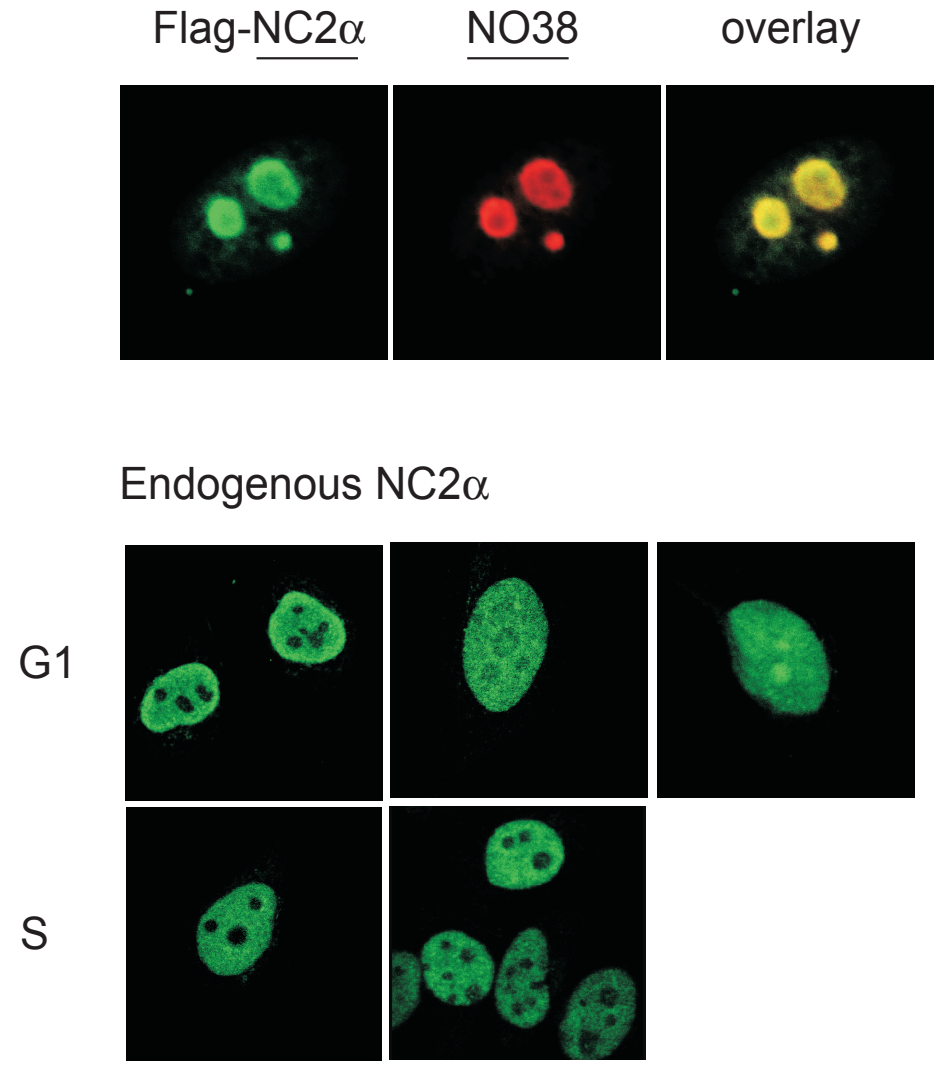


Figure 9. NC2α colocalizes with a nucleolar protein. (A) HeLa cells were transfected with Flag-NC2α and stained with ab against NC2α and the nucleolar specific protein NO38. The overlay of the staining clearly shows the colocalization of the two proteins. (B) HeLa cells were blocked in the G1 and S phases of the cell cycle and analyzed by immunofluorescence as described in Fig. 8a. Endogenous NC2α is observed in the nucleoli only during the G1 phase, while it is excluded during

concentrated on the Interphase, and in particular on the G1 and S phases.
To obtain a homogeneous population of cells undergoing these phases, HeLa cells were treated with specific cell cycle inhibitors. In particular, G1 block was obtained exposing exponentially growing cells to mimosine for 17 h, whereas S blocked cells were obtained treating them with a double thymidine block. The synchronized cells were fixed, immunostained and finally analysed with the confocal microscope. NC2α is never observed in the nucleoli of S blocked cells (Fig 9b). On the contrary, the late G1 population shows both situations, with some cells where NC2α was excluded from the nucleoli and cells where it was clearly detectable. Hence, this experiment indicates that the nucleolar localization of NC2α is restricted to the G1 phase.

1.1.3. Localization of NC2β

Since the polyclonal rabbit antibody normally used for western blot is not suitable for immunofluorescence, it was not possible to look at the localization of the endogenous NC2β subunit. Therefore, HeLa cells were transfected with the tag fused proteins Ig-NC2β or flag-NC2β and later stained with antibody recognizing the Ig (i.e. any secondary ab against the heavy chain of the human IgG) and the flag tag. The exogenous NC2β protein is strongly detected in the nucleus (Fig. 8c). Opposed to what is observed for NC2α, the overexpressed NC2β is always excluded from the nucleoli and shows a weak signal in the cytoplasm. Moreover, the two tags do not seem to influence the localization of the protein, because both fusion constructs give the same localization.

Thus, both NC2 subunits show a largely overlapping distribution, in the nucleoplasm, but they exhibit also specific patterns: NC2α can be found in the nucleoli, whereas NC2β is present in low concentrations in the cytoplasm.

1.2. Import and dimerisation of NC2

1.2.1. Both NC2α and NC2β contain an NLS

The localization studies of NC2α and NC2β show that both subunits concentrate in the nucleus. This observation strongly suggests the presence of a nuclear localization signal (NLS) in both of them.
Indeed, the analysis of the amino acid sequences identified a putative, positively charged motif in each protein (Fig. 2b). NC2α contains a potential bipartite NLS in the N-terminus (4-KKKKYNARFPPARIKKI-20), composed of two clusters of basic

amino acids, mostly lysines, separated by a short linker. NC2β has only one cluster of basic amino acids, containing two lysines and two arginines (100-KRRK-103). The two putative sequences were tested using NC2 constructs potentially deficient for nuclear import.

The localization of His-NC2α, containing a small 6His N-terminal tag, was compared with that of Ig-NC2α, which has a big fusion of ca. 30kD at the N-terminus that could impair the close putative bipartite NLS. Cells transfected with the corresponding plasmids were stained with the NC2α ab and analyzed by confocal microscope.

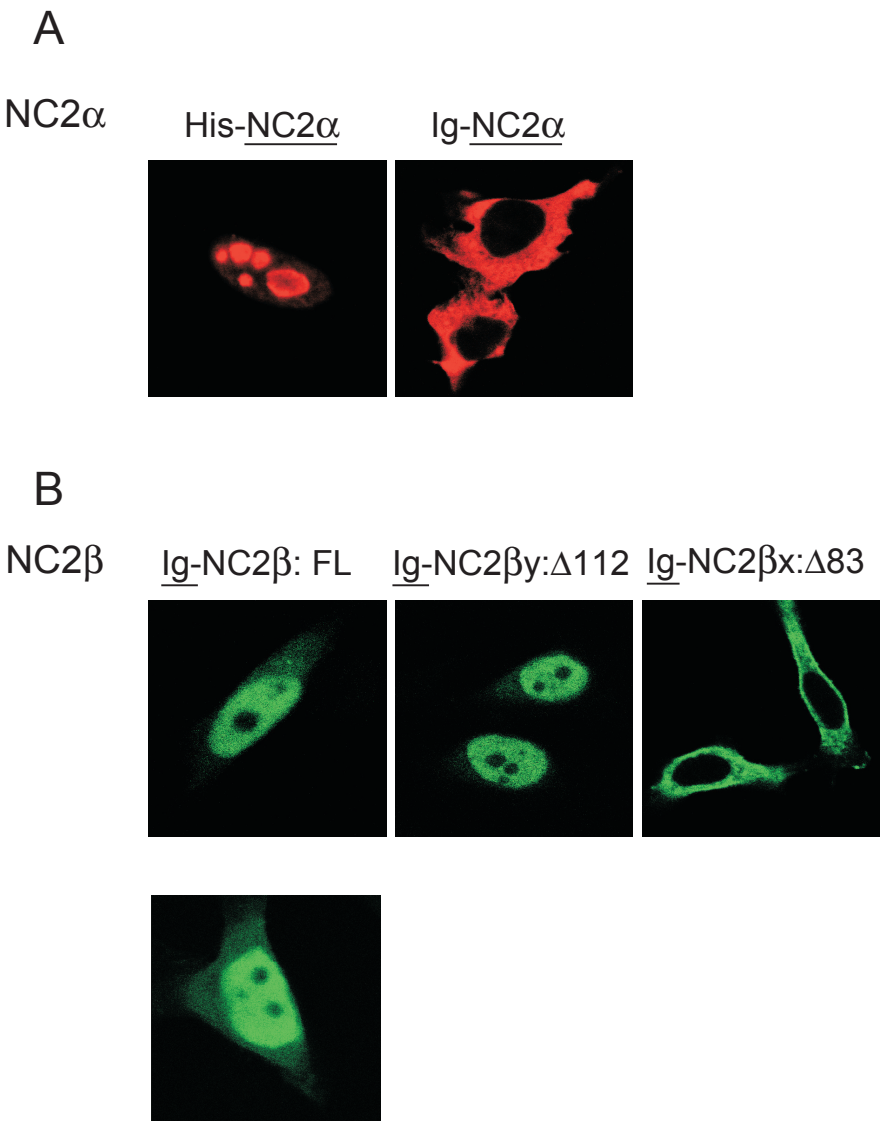
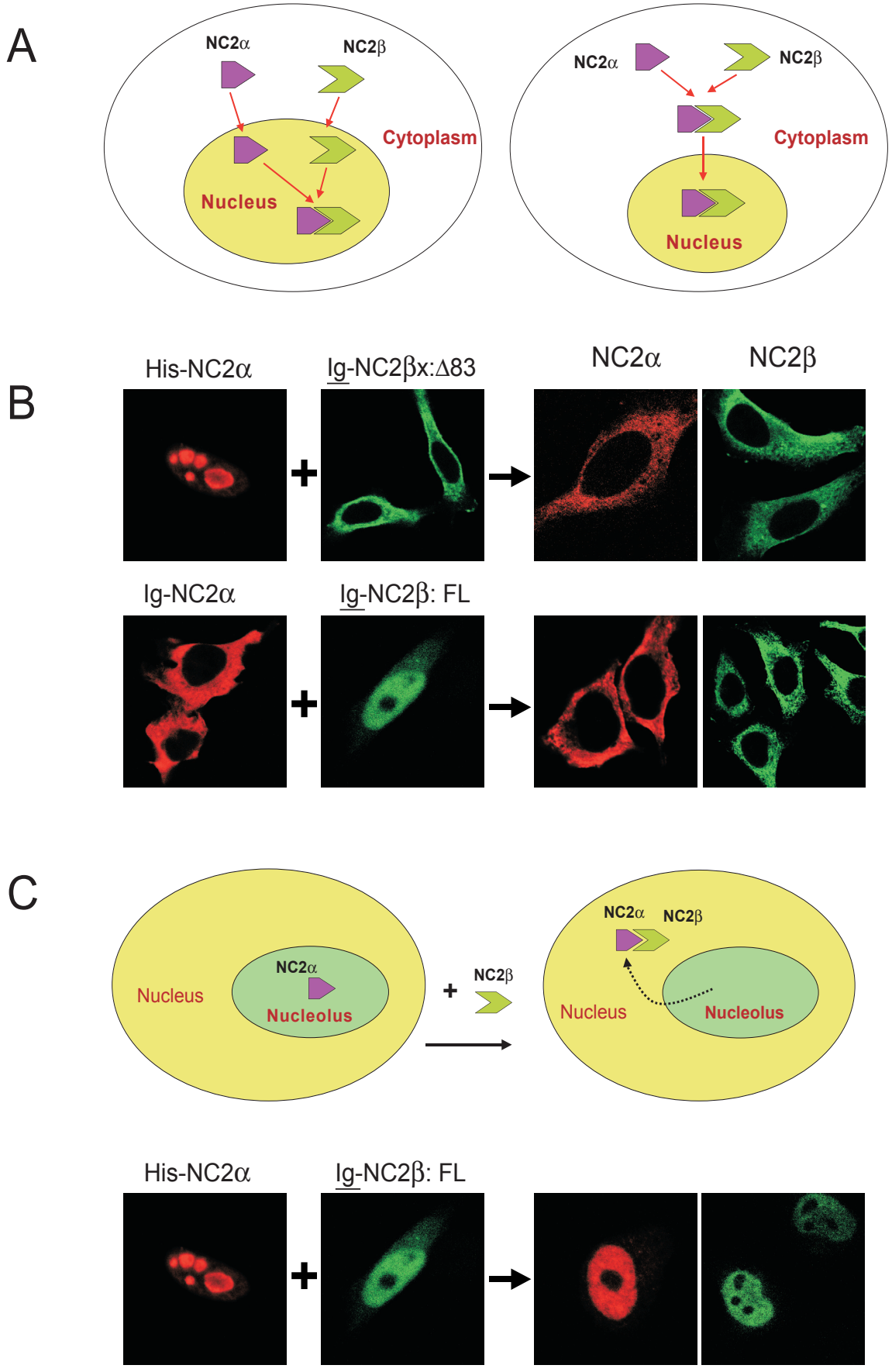


Figure 10. Both NC2 subunits contain a nuclear localization signal (NLS). (A) HeLa cells were transfected with two different NC2α constructs, Ig-NC2α and His-NC2α. The fusion of the big Ig-tag, adjacent to the putative NLS, impairs transport to the nucleus. (B) Transfection of HeLa cells with successive deletion mutations of NC2β. Only full length NC2β (Ig-NC2β:FL) and deletion construct containing the first 112 aa (Ig-NC2β:D112) accumulates in the nucleoplasm. In contrast, the short deletion version (Ig-NC2β:D83) is retained in the cytoplasm, indicating the presence of a NLS between aa 84 and 112.



See next page for the legend.

As expected, His-NC2α localizes in the nucleus and accumulates into the nucleoli. In contrast, the big Ig-NC2α fusion protein is totally excluded from the nucleus (Fig. 10a). This can be explained if we assume that in the Ig-NC2α fusion the NLS is not functional any more, and the large size prevents the construct to enter the nucleus by diffusion. Probably, the NLS is masked by the steric hindrance of the large proximal Ig fusion. As a consequence, the NLS would not be exposed on the surface of the protein, precluding its recognition by the nuclear import machinery.

The localization of the Ig-NC2β construct, normally imported into the nucleus, was compared with that of two successive deletion mutants, the Ig-NC2βy (amino acids 1-112), still containing the potential NLS sequence (which is located between 99-103), and the Ig-NC2βx deletion (amino acids 1-83) lacking the putative nuclear import signal. HeLa cells were transfected, stained with an ab. directed against the Ig tag and the distribution of the proteins was observed in the microscope. As expected, both Ig- NC2β and Ig-NC2βy accumulate in the nucleus. In contrast, the Ig-NC2βx deletion mutant is completely excluded from the nucleus (Fig. 10b). This result demonstrates that the sequence comprised between amino acids 84 and 112 contains a nuclear targeting sequence, which is necessary and sufficient for nuclear transport of NC2β. In this region, the only motif having the requirements for a nuclear targeting sequence is the cluster of positively charged aminoacids 100-KRRK-103, which therefore may be regarded as the most likely NLS candidate.

1.2.2. Is NC2 transported into the nucleus as a dimer or as single subunits?

The discovery of NC2 constructs deficient in nuclear import offered us a powerful tool to investigate the modality of transport of NC2 into the nucleus. One could think of two different scenarios: (i) NC2α and NC2β dimerize in the cytoplasm immediately after translation and then they are imported in the nucleus as a dimer; (ii) they enter the nucleus as single subunits, thanks to specific NLS, and they dimerize only in the nucleus (Fig. 11a).

To address this question, double transfection experiments were performed combining a construct normally localizing into the nucleus with a construct of the

---

Figure 11 (previous page). Import and dimerization of NC2. (A) Two models for the dimerization of NC2 subunits are showed: (i) NC2α and the partner NC2β dimerize after entering into the nucleus independently; or (ii) they enter into the nucleus as a dimer. (B) HeLa cells were double transfected with one subunit lacking the nuclear import functionality (see Fig. 19 and text for details) and the other subunit as wild type. Both subunits are retained in the cytoplasm whenever one lacks the ability to enter the nucleus, supporting model (i). (C) In the nucleus, NC2β controls the localization of both subunits (as represented in the scheme). Cotransfection of HeLa cells with His-NC2α and Ig-NC2β abolishes the normal nucleolar concentration of NC2α, suggesting that NC2α is present in the nucleoli only when in excess as single subunit compared to NC2β.

other subunit lacking the import function. Surprisingly, both subunits were retained in the cytoplasm independently of whether NC2β or NC2α lacked a functional NLS (Fig. 11b). Moreover when His-NC2α is cotransfected with Ig-NC2β, NC2α does not localize in the nucleoli any longer, but stays in the nucleoplasm together with NC2β (Fig. 11c).

1.3. Characterization of the NLS

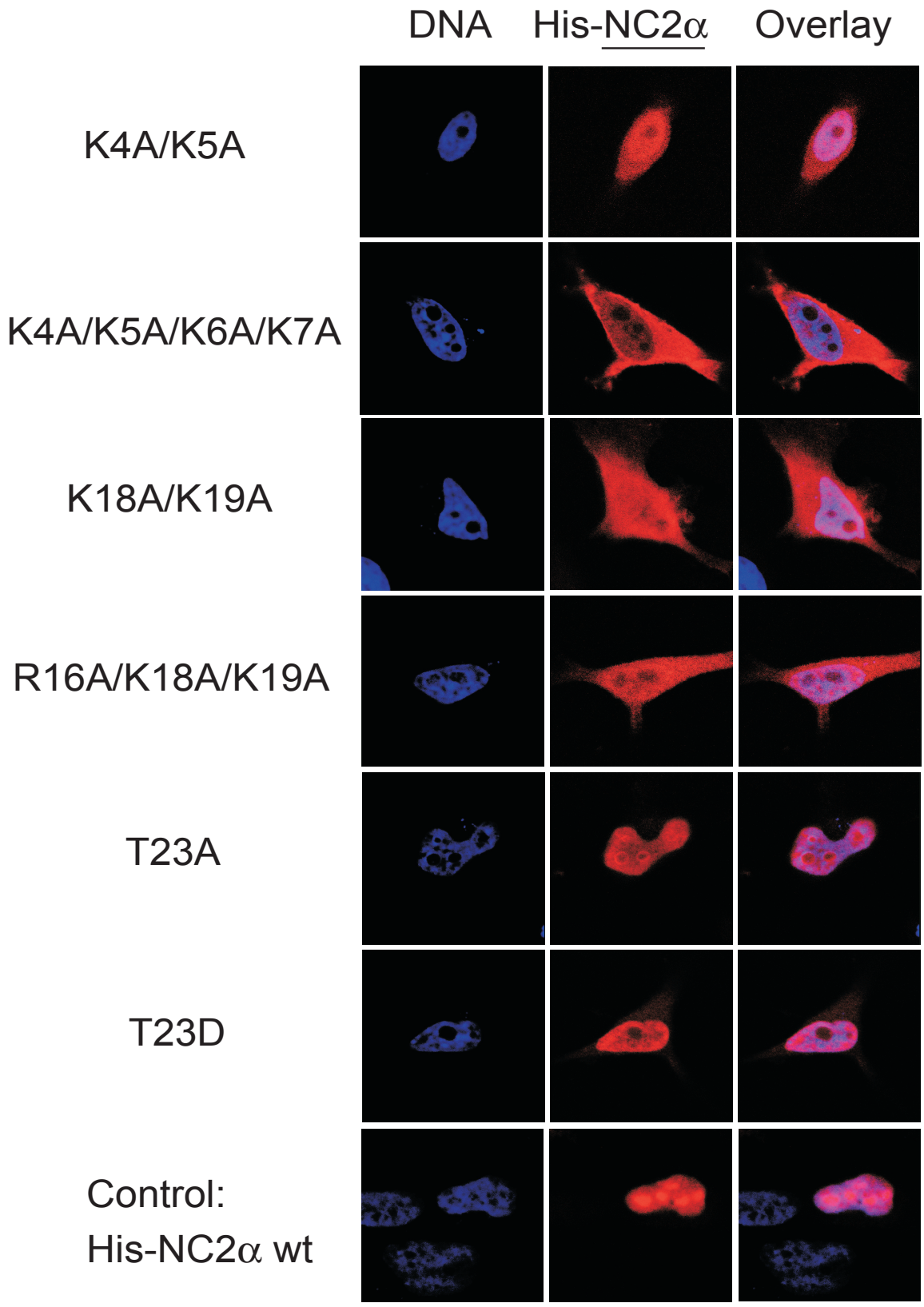
1.3.1. NC2α has a bipartite NLS

In order to investigate the role of the hypothetical bipartite NLS of NC2α, mutants were generated in both putative NLS motives of the His-NC2α vector (4-KKKKYNARFPPARIKKI-20). In the first cluster of basic amino acids, either only lysines 4-5 (K4A/K5A) or the whole positive stretch of lysines (K4A/K5A/K6A/K7A) were converted into alanine. In the second NLS motif, the two lysines (K18A/K19A) were converted into alanine and, in a second cloning procedure, also the arginine was mutated into alanine (R16A/K18A/K19A). As control, the vectors His-NC2α, for nuclear transport, and Ig-NC2α, for deficiency in nuclear import, were also included. Interestingly, all mutations affected the import: none of the mutants localized into the nucleus as exclusively as the wild-type protein, although the cytoplasmic localization is never as strong as in the control Ig-NC2α (Fig. 12). Probably, His-NC2α mutants can enter the nucleus also by diffusion, resulting in the observed spread distribution, whereas the large Ig-NC2β fusion protein can pass through the nuclear pore only by active transport and not by diffusion. Regarding the relative strength of the two NLS, mutations in the second sequence were less dramatic than those in the first one. All the mutations in the second NLS cause a diffuse distribution throughout the cell (Fig. 12). The substitution R16A/K18A/K19A caused

Figure 22 (next page). NC2α has a bipartite N-terminal nuclear localization signal (NLS). HeLa cells were transfected with different His-NC2α constructs, mutated in the putative NLS, and visulalized with NC2α ab and secondary ab Cy3-conjugated. Left column shows DNA stained with DAPI, central column the NC2α staining, the right the overlay. The His-NC2α construct (i.e. the wt control) was mutagenized in the two motifs of the putative N-terminal NLS (shown in the on the top of the panel): underlined amino acids (aa) correspond to the putative bipartite NLS; aa in bold are the mutated ones (their position relative to the NC2α start site is shown above the sequence). Mutants are named after the mutated amino acid/s: the wild type aa precedes the position number and the aa it was mutagenized to (e.g. K4A/K5A: lysines 4 and 5 were both mutated to alanine). Mutations of either one of the motifs affect nuclear import (K4A/K5A, K4A/K5A/K6A/K7A, K18A/K19A, R16A/K18A/K19A). Mutant K4A/K5A/K6A/K7A (comprising the whole first motif) is the one impairing more severely nuclear import. When threonine 23 was mutated to aspartate (T23D), mimicking its potential phosphorylation state, nucleolar localization was abolished (cfr. T23D and T23A, where threonine was mutated to the neutral alanine).

4 5 6 7 16 18 19 23

4-**KKKK**YNARFPPA**RIKK**IMQ**T**-23



See previous page for the legend.

a reduction in nuclear targeting no greater than that observed for the change K18A/K19A, suggesting that R16 does not play a major role.
More evident are the effects of mutations in the first motif. The substitution K4A/K5A affects only marginally nuclear import, and the protein still concentrates in the nucleus. However, the mutant K4-7A remains mainly in the cytoplasm, with only a small fraction entering the nucleus, most likely by diffusion.
Thus, both N-terminal motifs are necessary for targeting NC2α to the nucleus, but the first signal is the strongest one. Notably all mutations impaired the ability of the protein to accumulate into the nucleoli.

1.3.2. Mutation of threonine 23 in aspartate abolishes nulceolar localization

NC2α contains potential phosphorylation sites close to the bipartite NLS (3-**S**KKKKYNARFPPARIKKIMQ**T**-23). Threonine 23 (T23) is conserved in yeast and previous studies showed that phosphorylation at this residue is important for NC2 transcriptional repression activity in yeast (Creton et al. 2002). We asked whether this observation was related to the fact that T23 is adjacent to the NLS and mutated this residue into alanine and aspartate, to mimic the negative charge acquired by phosphorylation. As expected, the His-NC2α-T23A mutant shows exactly the same distribution of the His-NC2α wild type, with signal associated to the nucleoplasm and especially to the nucleoli (Fig. 12). Interestingly, the His-NC2α-T23D mutant still accumulates in the nucleoplasm, but does not enter the nucleoli.

1.3.3. NC2β has a single motif NLS

The analysis of the localization of the Ig-NC2β deletion mutants suggests the sequence 100-KRRK-103 to be the NLS for NC2β. To confirm this hypothesis, this motif was partially mutagenized and the resulting subcellular localization studied. In a first experiment, the two central arginines were replaced with two uncharged alanines (R101A/R102A). In a second, both serines adjacent to the potential NLS (100-KRRKASSR-107) were mutated into alanine (S105A/S106A) or aspartate (S105D/S106D), to mimic the negative charge acquired after serine phosphorylation. In this latter experiment, we made use of the interesting observation that nuclear import of NLS cargo proteins can indeed be impaired by phosphorylation at sites within or adjacent to a classic NLS sequence (Jans 1995; Hood and Silver 1999; Jans et al. 2000).
All the mutations were introduced in the construct Flag-NC2β, because it is highly expressed in the cells and easily detectable with the anti-flag ab. As control, cells were transfected with non-mutated Flag-NC2β and Ig-NC2β (Fig. 13), which accumulate in the nucleus, and Ig-NC2βx, which is excluded from it. All mutations

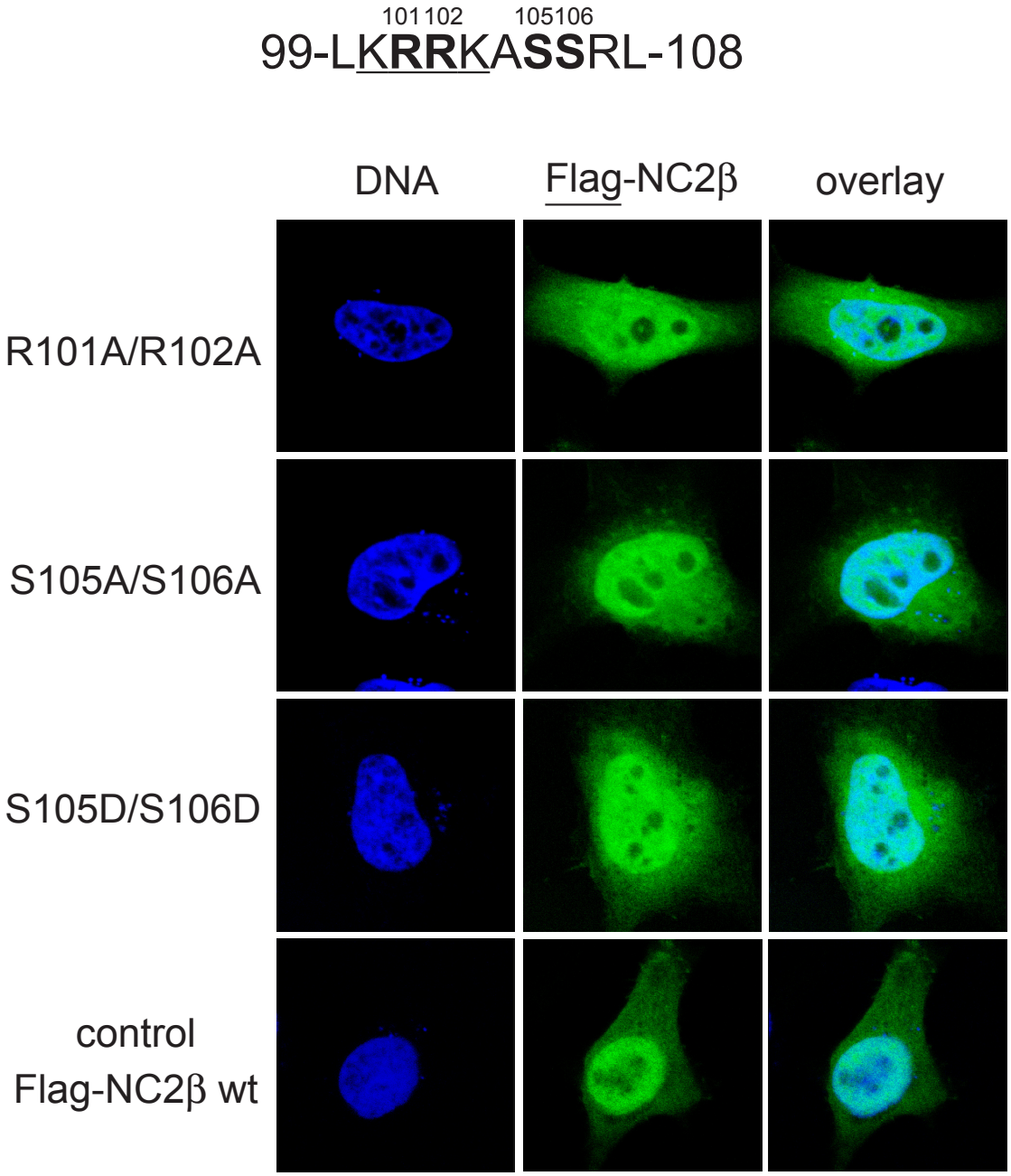


Figure 13. Mutagenesis of part of NC2β putative nuclear localization signal (NLS) does not affect its subcellular localization. HeLa cells were transfected with Flag-NC2β construct mutagenized in the putative NLS. NC2β was visualized with ab directed against the Flag-tag and a secondary ab FITC-conjugated. Left column shows DNA stained with DAPI, central column the NC2β staining, the right the overlay. The Flag-NC2β construct represents the wt control. The putative NLS is shown as underlined sequence on the top of the panel. The mutagenized aa are indicated as bold letters (their position relative to the NC2β start site is shown above the sequence). Mutants are named after the mutated amino acid/s (see Figure 22 legend for explanation). The putative NLS was mutagenized in the two central arginines (R101A/R102A). Also the adjacent (non-NLS) serines were mutagenized to alanine or aspartate (S105A/S106A and S105D/S106D, respectively) mimicking the effect of phosphorylation. All mutations resulted in a subcellular distribution similar to the control.

result only in a slightly larger amount of NC2β in the cytoplasm, as compared to the control flag-NC2β. This suggests that the two remaining lysines of the NLS are able to drive the interaction with the nuclear import receptor and that the two central arginines are not critical for the import. Moreover a negative charge at positions 105 and 106 may affect only moderately NC2β import.

1.3.4. Only the first motif of the NC2α-NLS is important for import of the dimer

To assess the effect of NC2α and NC2β NLS mutations on nuclear targeting of the dimer, HeLa cells were transfected with both subunits, wild type or mutated (Fig. 14). In the control experiment, the cells were transfected either with both proteins going into the nucleus (His-NC2α and Flag-NC2β) or with one wt subunit (His-NC2α, Ig-NC2β) together with the construct lacking the nuclear targeting function (Ig-NC2α, Ig-NC2βx). As shown before, if both proteins have a functional NLS (His-NC2α and Flag-NC2β), the dimer localizes in the nuclei. Both signals are necessary because if one of them is missing (Ig-NC2βx) or is not functional (Ig-NC2α), NC2 accumulates in the cytoplasm. To test the ability of the mutants to impair nuclear transport of the dimer, Flag-NC2β was cotransfected together with the construct lacking either the first lysine stretch (K4A/K6A/K7A) or the second motif (R16A/K18A/K19A) of the bipartite NC2α NLS. The analysis at the confocal microscope showed that when the second part of NC2α-NLS is missing the dimer accumulates in the nucleus as efficiently as the control, indicating the first NLS to be sufficient for correct targeting of the dimer. In fact, when the whole lysine stretch in the first motif is replaced by alanine, NC2 distributes all over the cell, implying that the first NLS motif is sufficient and also necessary for the correct transport of NC2.
Thus, if both motifs are important for the nuclear localization of the single NC2α subunit, only the first one is necessary for the import of the dimer.

1.3.5. Mutation of the NC2β-NLS affects import of the dimer

When HeLa cells were cotransfected with His-NC2α wt and the Flag-NC2β-R101A/R102A mutant, part of the dimer is localized in the cytoplasm (Fig. 14). This was surprising, because the R101A/R102A mutation did not have a strong effect in nuclear import of NC2β alone, and suggests that arginines 101-102 indeed play a role in nuclear transport.
To investigate the effects of serines 105-106 phosphorilation on the nuclear import of the dimer, both Flag-NC2β-S105A/S106A and Flag-NC2β-S105D/S106D mutants were cotransfected with His-NC2α wt. In both double transfection experiments the NC2 mutants concentrated in the nucleus indifferently from the charge of the

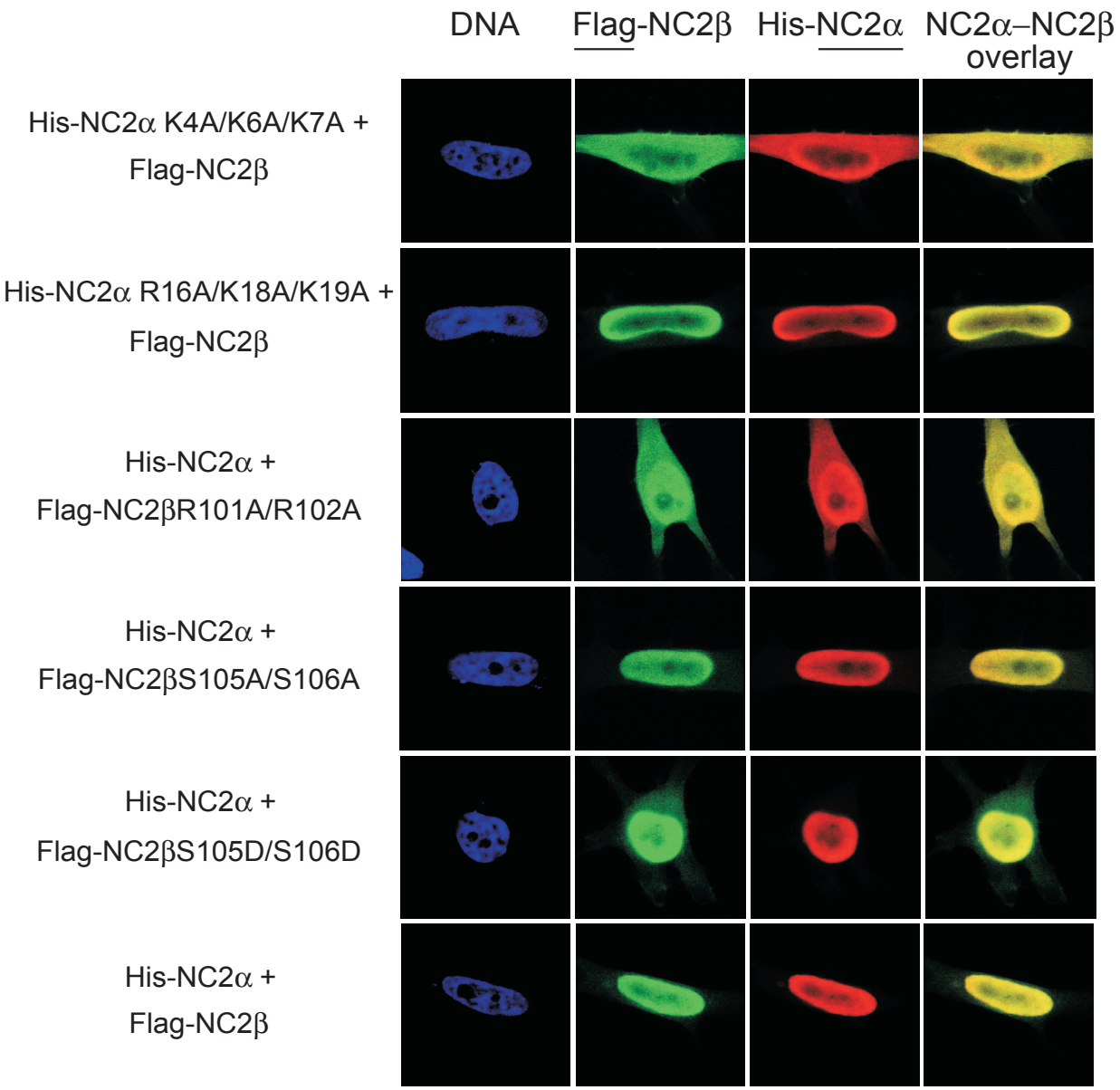


Figure 14. Both NC2α and NC2β NLSs are essential for nuclear localization of the NC2 dimer. HeLa cells were double transfected with His-NC2α or Flag-NC2β wt or mutated and stained as described before. First column shows DNA stained with DAPI, second column the NC2β staining, third column the NC2α staining, and the fourth the overlay of the two subunits. The His-NC2α construct (i.e. the wt control) mutagenized either in the whole first or second motif (K4A/K5A/K6A/K7A or R16A/K18A/K19A, respectively) was cotransfected with the Flag-NC2β wt. Vice versa, the Flag-NC2β mutants (R101A/R102, S105A/S106A and S105D/S106D) were co-transfected with the His-NC2α wt. Only the first motif of the bipartite NC2α NLS is important for import of the dimer. Mutation of the two central arginines of the NC2β NLS also affects localization of the dimer, although it did not seem to be important for transport of the single subunit.

two serines, showing essentially the same distribution pattern as that seen in the control. Thus, phosphorylation at these residues may have a weak effect on the single subunit but not on the dimer.

2. Post-translational modification of NC2: NC2α is specifically hyperphosphorylated during mitosis

2.1. NC2 is neither acetylated nor methylated

Post-translational modifications play an important role in the regulation of protein activity. In order to investigate whether NC2 is post-translationally modified by methylation and/or acetylation, recombinant NC2 was incubated with HeLa nuclear extracts, which contain acetyl- and methyl-transferase activities. Radioactively labeled Acetyl-Coenzyme A and S-Adenosylmethionine were added to the reaction, as donors of radioactive acetyl and methyl groups, respectively. As

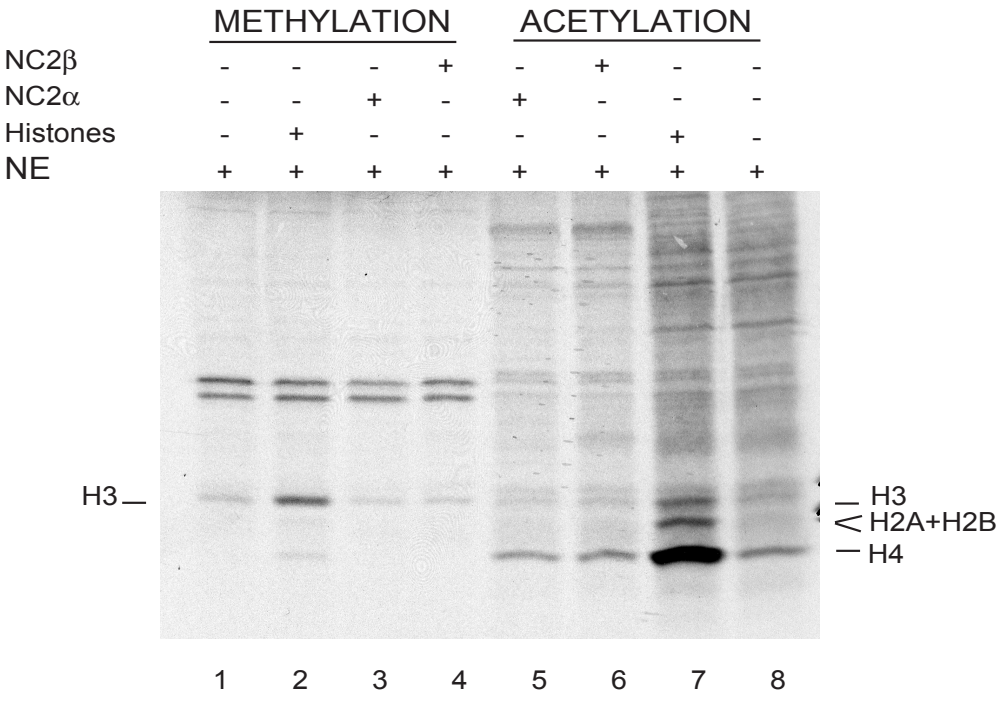


Figure 15. NC2 subunits are neither methylated nor acetylated. Recombinant NC2 (3 mg of each subunit) and recombinant histone proteins (3 μg) were incubated with 10 μl of HeLa nuclear extracts and [14C]-Acetyl-Coenzyme A and [14C]-S-Adenosylmethionine. After reaction, proteins were separated in a SDS-PAGE and visualized by autoradiography. Lanes 1-4 correspond to methylation assay, lanes 5-8 to acetylation assay. In contrast to histones (H2A, H2B, H3 and H4), no band corresponding to NC2 proteins was observed.

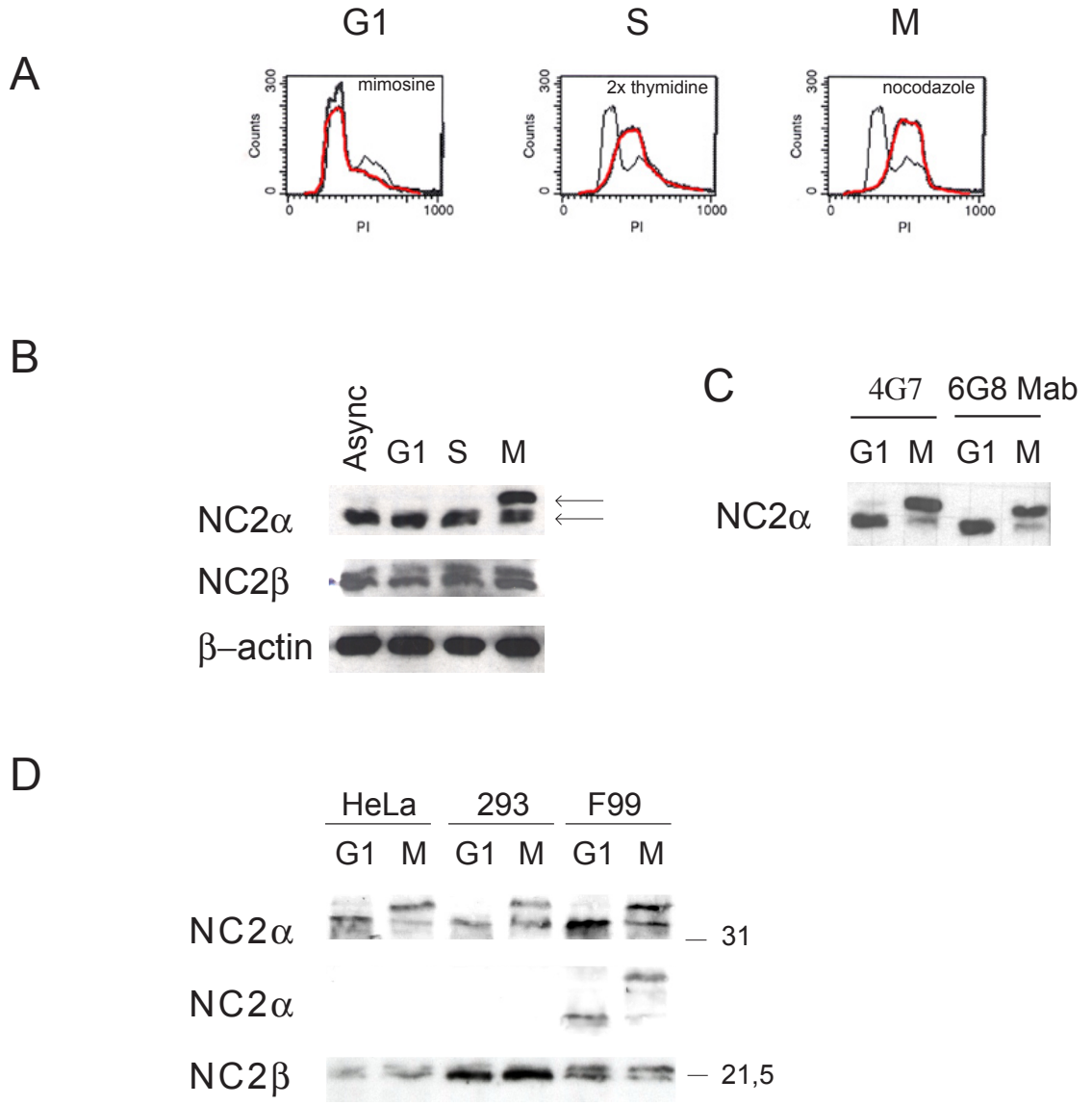


Figure 16. Levels of NC2 during the cell cycle: the NC2α subunit is modified during mitosis. (A) Analysis by flow cytometry of the DNA content (DNA is stained with propidium iodide, PI) of HeLa cells synchronized in different cell cycle phases (G1, S and M, see methods), compared to an asynchronous population. (B) Analysis by immunoblotting of the amount of NC2α and NC2β during the cell cycle. Whole cell extracts (WCE) were prepared from HeLa cells exponentially growing (Async) and synchronized (G1, S, M, see methods). The proteins corresponding to the same number of cells were separated in SDS-PAGE and immunoblotted with NC2α and NC2β antibodies. Immunostaining with the anti-β-actin ab was included as control for load of equal protein amounts. Protein levels are maintained constant through the cell cycle, but NC2α is modified in mitotic cells. (C) Analysis by immunoblotting of NC2α in cells synchronized in the G1 and M phases of the cell cycle. The membrane was immunostained with two different monoclonal antibodies against NC2α (4G7 and 6G8). The additional NC2α band observed in mitosis is recognized by both antibodies, showing that this band is indeed NC2α and not a cross reaction of the 4G7 antibody with a mitotic protein. (D) Immunoblot analysis of WCE prepared from different cell lines, synchronized in the G1 and M phases of the cell cycle. HeLa and 293 are transformed cell lines, whereas F99 are skin primary fibroblasts. The 293 WCE were obtained resuspending the cells in lysis buffer, like for the other cells type, but then cells lysates were also sonicated. The proteins corresponding to the same number of cells were separated in SDS-PAGE. The mitotic specific NC2α band is observed in all the cell lines. The F99 extracts contain an extra NC2α band running between the 21.5 kD and 30 kD marker bands, recognized by both NC2α monoclonal antibodies (data not shown). This band is

control, recombinant histone proteins were included, since they are known to be typical substrates for acetyl- and methyl-transferases. Proteins were separated electrophoretically and revealed by autoradiography of the gel. None of the radioactive bands observed in the autoradiography corresponded to NC2α or NC2β, indicating that these proteins were not modified (Fig. 15).

2.2. Monitoring of NC2 level throughout the cell cycle: a new isoform of NC2α appears during mitosis (M-NC2α)

So far there are no indications about the level of the two NC2 subunits throughout the cell cycle. Thus, we asked whether the amount of the proteins was maintained constant, or was changing depending on a specific phase of the cell cycle. To address this question, HeLa cells were synchronized in the G1, S and M phases of the cell cycle, using different inhibitory compounds (Fig. 16). The correct synchronization of the cells was verified by flow cytometry (FACS) analysis of a parallel culture (Fig. 16a). Whole cell extracts (WCE) were prepared from the cells, run in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed in Western Blot (WB). Staining of the β-actin was used as a control for equal load of total proteins amount in the different lanes. NC2α was detected with the rat 4G7 monoclonal antibody, and NC2β with a rabbit polyclonal ab (Fig. 16b). Analysis of the WB indicated that NC2β did not show any variation in protein level or in migration in the different WCE preparations. The same was observed for NC2α in the G1 and S phases. Surprisingly, during the M phase, an additional slower mobility form of NC2α appears. To confirm that this extra band was specific and not the result of a cross reaction of the antibody, a new blot carrying G1 and M WCE was probed with two monoclonal NC2α antibodies (4G7 and 6G8; Fig. 16c). Both antibodies recognized the low mobility band, thus confirming that NC2α undergoes mitosis-specific modification.

2.3. The new M-NC2α form is specific for the mitotic stage

To prove that M-NC2α is characteristic only for the M phase, mitotic HeLa cells were released from the block and allowed to enter G1 in normal medium. Furthermore, to test if M-NC2α was a result of a phosphorylation, the release was performed either in presence or absence of the phosphatase inhibitors NaF and Vanadate. Asynchronous and mitotic cells, and cells collected at 6h from the mitotic release, were used to prepare the WCE. An aliquot of each sample was used to establish the cell cycle stage by FACS (Fig. 17a). Unexpectedly, the flow cytometry analysis

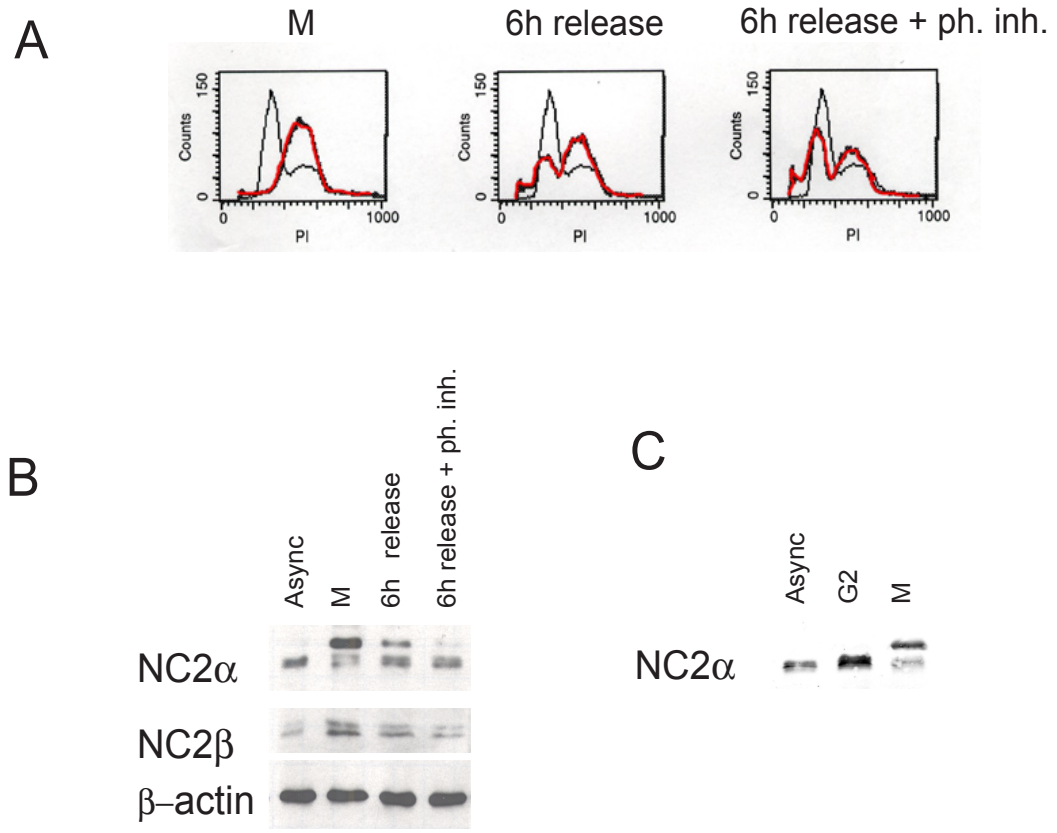


Figure 17. NC2α modification is restricted to mitosis. (A) FACS analysis of the DNA content (PI) of HeLa cells synchronized in M phase with nocodazole and released from the block for 6 h in the absence or presence of phosphatase inhibitors (ph. Inh., see methods), compared to an asynchronous population. (B) Immunoblot analysis of WCE prepared from the cells populations described in A. The mitotic specific form disappears when the cells exit mitosis. (C) NC2α was analyzed in immunoblot from WCE prepared from asynchronous cells, and cells blocked in G2 and M. NC2α is modified only once cells enter mitosis.

revealed an acceleration in the release from the M phase in presence of phosphatase inhibitors. This is in line with the parallel immunoblot experiment: M-NC2α was still observed at 6h post-release, but if phosphatase inhibitors were present, most of the cells entered the G1 phase and M-NC2α was almost totally gone (Fig. 17b). Hence, these results confirm that the low mobility form of NC2α is characteristic of the mitotic phase.

It remained to be shown whether the modification of NC2α occurs specifically in mitosis, or rather in late G2. To test this, HeLa cells were synchronized at late S phase by double thymidine block and released in normal medium for 8h to let them reach the G2 stage. Flow cytometry analysis of a parallel culture confirmed the correct synchronization of the cells in the G2 and M phases compared to the control

sample with asynchronous cells (data not shown). WCE from the three samples were fractionated by SDS-PAGE and examined by immunoblot experiments with NC2α and NC2β antibodies (Fig. 17c). The results showed that NC2α is modified in cells blocked in mitosis but not in late G2, suggesting that an enzyme specifically activated in mitosis is responsible for the modification.

2.4. M-NC2α is observed in different cell types

To exclude the possibility that this observation was exclusive of the cell type or of transformed cells in general, different types of transformed cells available in the lab (HeLa suspension and adherent, 293) and skin primary fibroblast (F99) were blocked either in G1 or in M phase. Immunoblot analysis revealed the presence of M-NC2α in all above cell types (Fig. 16d), indicating that this is a general phenomenon. Again, no difference concerning NC2β was observed (data not shown).

Interestingly, in the F99 primary cells WCE the NC2α ab recognizes another band, which is smaller than the usual one and has mobility between the 21 and 31 marker bands. Surprisingly, also this high mobility band seems to be modified in mitosis. Both the NC2α monoclonal antibodies recognize this high mobility band. Since the epitopes of these antibodies are mapping the C-terminus of NC2α, this band is likely to correspond to a N-terminal deleted variant.

2.5. M-NC2α is phosphorylated

2.5.1. In vitro dephosphorylation of the mitotic NC2α

The shift in mobility observed in WB of the mitosis-specific NC2α form is compatible with a modification by phosphorylation. To test this assumption, HeLa mitotic WCE were incubated with the λ phosphatase enzyme, resolved by SDS-PAGE and analyzed in WB (Fig. 18a). Interestingly, the phosphatase treatment not only abolished the mitotic shift, but also produced a band with higher mobility (lanes 4 and 5) than the regular NC2α band observed in the G1 sample (lane 1). The change in mobility observed in lanes 4 and 5 is specifically produced by the λ phosphatase because treatment with the enzyme inhibitor, the vanadate, preserved phosphorylation throughout the incubation (compare lanes 6 with 4 and 5).

2.5.2. In vivo phosphorylation of NC2

The previous experiment is an indirect proof that the modification responsible for

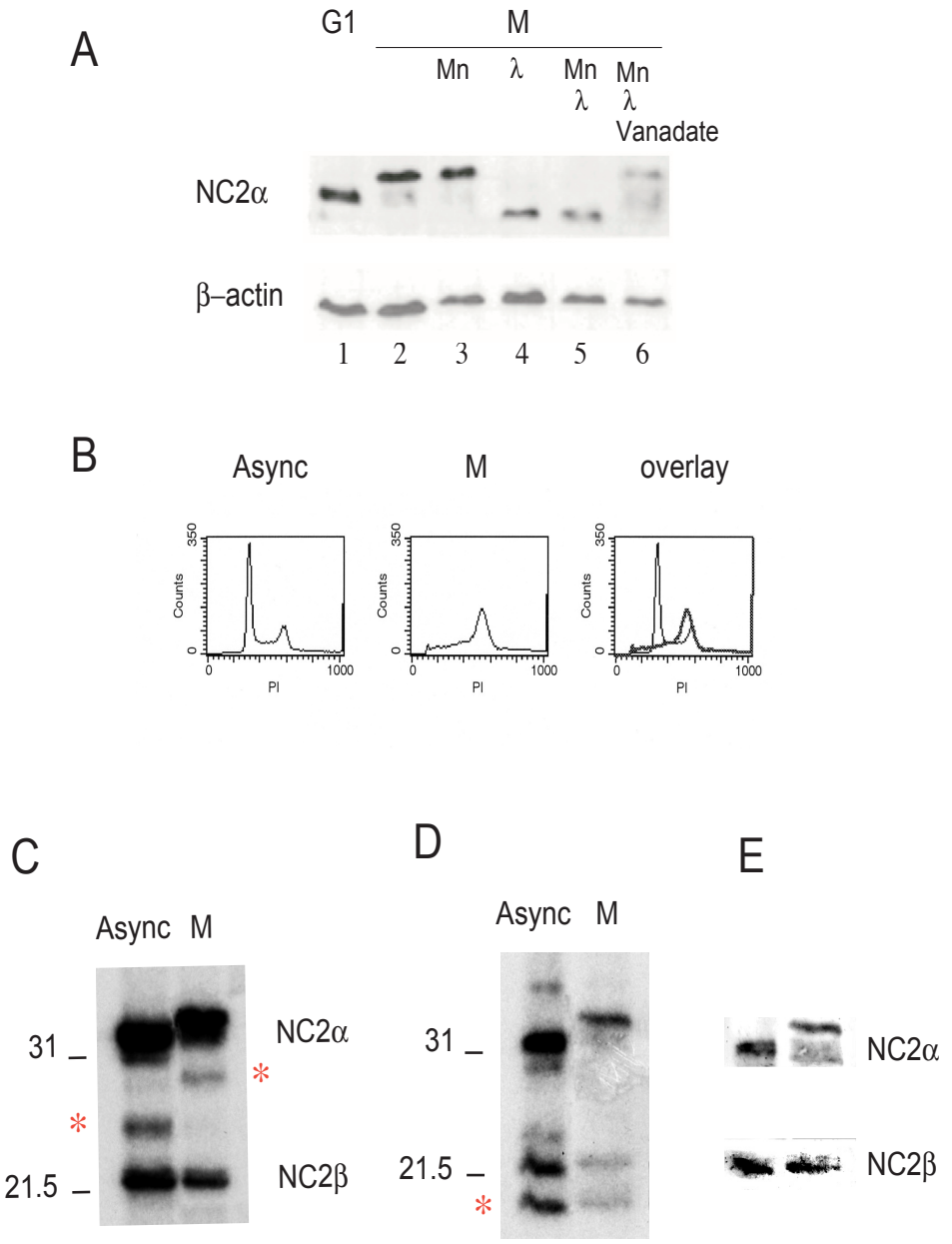


Figure 18. NC2α is modified by phosphorylation during mitosis. (A) Mitotic HeLa WCE were treated with the λ phosphatase enzyme alone or in the presence of either Manganese (Mn) or Mn and Vanadate, a phosphatase inhibitor. The reaction was carried out for 30 min at 30°C. The extracts were then separated in SDS-PAGE and immunoblotted with the NC2α antibody. As controls, untreated G1 extracts were included in the first lane and the blot was also probed with the β-actin antibody. Phosphatase treatment produces an NC2α band with mobility even higher than the normal G1-NC2α band (compare lanes 4, 5 and lane1). (B-E, C, D) In vivo labeling analysis of NC2α and NC2β with [32P]H3PO4 in asynchronous and M-blocked HeLa cells (see methods). (B) Flow cytometry analysis of the DNA content of the two cells populations. Radioactive NC2 was immunoprecipitated with the NC2α antibody and then proteins were eluted from the antibody with two methods. (C) Autoradiography of the gel made with the peptide eluted proteins. (D) Autoradiography of the gel made with the SDS eluted proteins. (E) The gel corresponding to the SDS elution was also transferred to a nitrocellulose membrane and probed with the NC2α and NC2β antibodies. Both NC2 subunits are phosphorylated in vivo and NC2α undergoes mitosis specific phosphorylation. In the autoradiography, additional bands are visible (red mark), compared to the Western Blot.

the change in mobility of M-NC2α is a phosphorylation. To test the possibility that mitosis produces a change in the overall phosphorylation status of NC2α, HeLa cells were metabolically labeled with [^{32}P]H_3PO_4 while exponentially growing or during the last 5h of the nocodazole treatment (mitosis block). A parallel culture of non-radioactive labeled HeLa cells treated in the same way was used for the flow cytometry analysis of the asynchronous (Async) and the mitotic blocked (M, Fig. 18b) cells.

The two different samples of HeLa cells metabolically labeled (Async, and M) were lysed in the presence of protease and phosphatases inhibitors. NC2 was immunoprecipitated with the NC2α 4G7 Mab and eluted first by way of a peptide corresponding to the epitope of the ab. and then with 2x SDS sample buffer. The two immunoprecipitates were separately fractionated by SDS-PAGE, blotted to NC membrane, and exposed for autoradiography before immunodetection of NC2α and NC2β. Autoradiography revealed that all NC2α and NC2β forms normally detected in WB are *in vivo* phosphorylated in interphase and especially in mitosis (Fig. 18c-e), confirming the data of the *in vitro* dephosphorylation assay. Thus, this is a direct proof that the modification responsible of the upper mobility shift of NC2α during mitosis is a phosphorylation.

Comparison of the two autoradiographies revealed the presence of additional

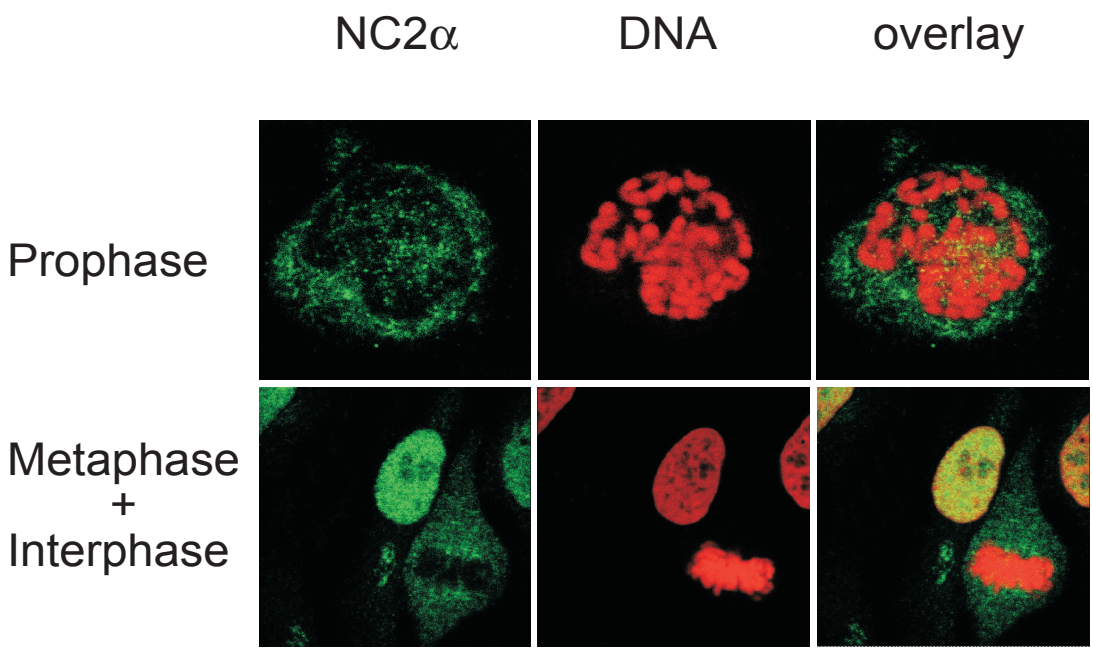


Figure 19. Subcellular distribution of NC2α during mitosis. HeLa cells were blocked in mitosis with nocodazole, NC2α was stained as described before. Left column shows DNA stained with PI, central column the NC2α staining, the right one the overlay. In interphase the staining is confined into the nucleus; in prophasic cells most of NC2α is not localized in the chromosomal regions, while in metaphase cell showed diffusely stained cytoplasm.

isoforms normally not recognized in WB (Fig. 18e). In particular, in the SDS elution there is an additional high mobility band running below the 21kD marker (asterisk in Fig. 18d). Additional experiments suggested that this band is an isoform of the NC2β subunit (see paragraph 4.1 of the results). Furthermore, an extra band (asterisk in Fig. 18c) running between NC2α and NC2β, which shows a change in mobility and is also phosphorylated during mitosis, has characteristics similar to the high mobility NC2α band observed in F99 cells (Fig. 16d). Presumably, this variant is normally not detected in immunoblot of HeLa extracts because it is very rare, while it becomes visible in the autoradiography due to the high intensity of the label.

2.6. NC2α redistributes from interphase nuclei to mitotic cytoplasm

To investigate the potential consequences of this hyperphosphorylation, the mitotic localization of endogenous NC2α was studied. Nocodazole blocked HeLa cells were fixed and incubated with the antibody against NC2α. DNA was stained with Propidium Iodide (PI) and cells were analyzed by confocal microscope (Fig. 19). As described before, immunofluorescent staining of NC2α in interphase cells is confined to the nucleus, with a finely speckled appearance. When the mitotic characteristic chromosome condensation has taken place and nuclear envelope breakdown has occurred, the mitotic cytoplasm is brightly stained, indicating that NC2 has redistributed throughout the mitotic cytoplasm. However, we cannot exclude that a minor part is still associated with mitotic chromosomes and overlooked in our immunofluorescence experiment because of the intensity of the staining of NC2α in the mitotic cytoplasm.

2.7. NC2α remains associated with NC2β also in its mitotically phosphorylated state

NC2α and NC2β form a very stable heterodimer through their histone fold domain. To investigate the possibility that the mitotic hyperphosphorylation of NC2α can affect dimer formation, NC2 was immunoprecipitated with the NC2α ab from asynchronous, G1 and mitotic blocked HeLa WCE. As shown in Figure 20a, NC2β coimmunoprecipitates with NC2α in all three extracts, indicating that mitotic NC2 remains intact. It is unlikely that NC2β coelutes exclusively with the weak interphase-NC2α band visible in the M lane, since the intensities of NC2β in M and G1 lanes are comparable. If NC2β coeluted with the weak interphase NC2α band, it would

not be detectable in WB, due to the very low sensitivity of the NC2β antibody. Notably, NC2α is depleted from all the extracts (see FT), but NC2β is not, suggesting that NC2β could be in excess compared to NC2α (Fig. 20a and 20c).

In a similar experiment, we checked if TBP was also coimmunoprecipitating with NC2α. TBP is coimmunoprecipitated with NC2 only from the mononucleosome fraction and never from nuclear extracts or WCE (Gilfillan et al., submitted paper). Preliminary data indicate that the C-terminal acidic region of NC2α could make additional contacts with TBP (Gilfillan, unpublished data). Therefore, since most of the potential phosphorylation sites of NC2α are located in the tail, we asked if the mitotic hyperphosphorylation strengthens the new potential TBP interaction domain, promoting NC2-TBP contact already in solution.
The WB membrane of the immunoprecipitation experiment was stained also with the TBP antibody (Fig. 20c). TBP never coimmunoprecipitated with NC2α, even in the mitotic WCE, suggesting that the addition of phosphorylated sites do not have appreciable effects on the interaction of the two proteins in solution.

2.8. Is M-NC2α excluded from the DNA?

2.8.1. Preparation of native NC2 and M-NC2 proteins for in vitro assays

The link between hyperphosphorylation and subcellular relocation of NC2α prompted us to investigate if the mitotic NC2 was still able to bind the DNA in a complex with TBP. This information can be obtained comparing the behavior of M-NC2 with that of the form observed in the other cell cycle phases. Native NC2 protein was immunopurified from WCE prepared from HeLa cells blocked in mitosis and interphase cells (*i.e.* asynchronous cells devoid of the mitotic ones, see Fig. 20b).

These two preparations were checked in WB to confirm that both subunits had been immunoprecipitated. As observed before, NC2β was detected in both samples (Fig. 20c).
To estimate the concentrations of NC2 in the peptide elutions and equalize them with the recombinant protein, I and M endogenous NC2 were titrated with increasing amount of recombinant protein (Fig. 20d). From the WB, the concentration of the endogenous NC2 was estimated to be between 5–10ng/□l. In addition, the active NC2 concentration was calculated in EMSA (see methods).

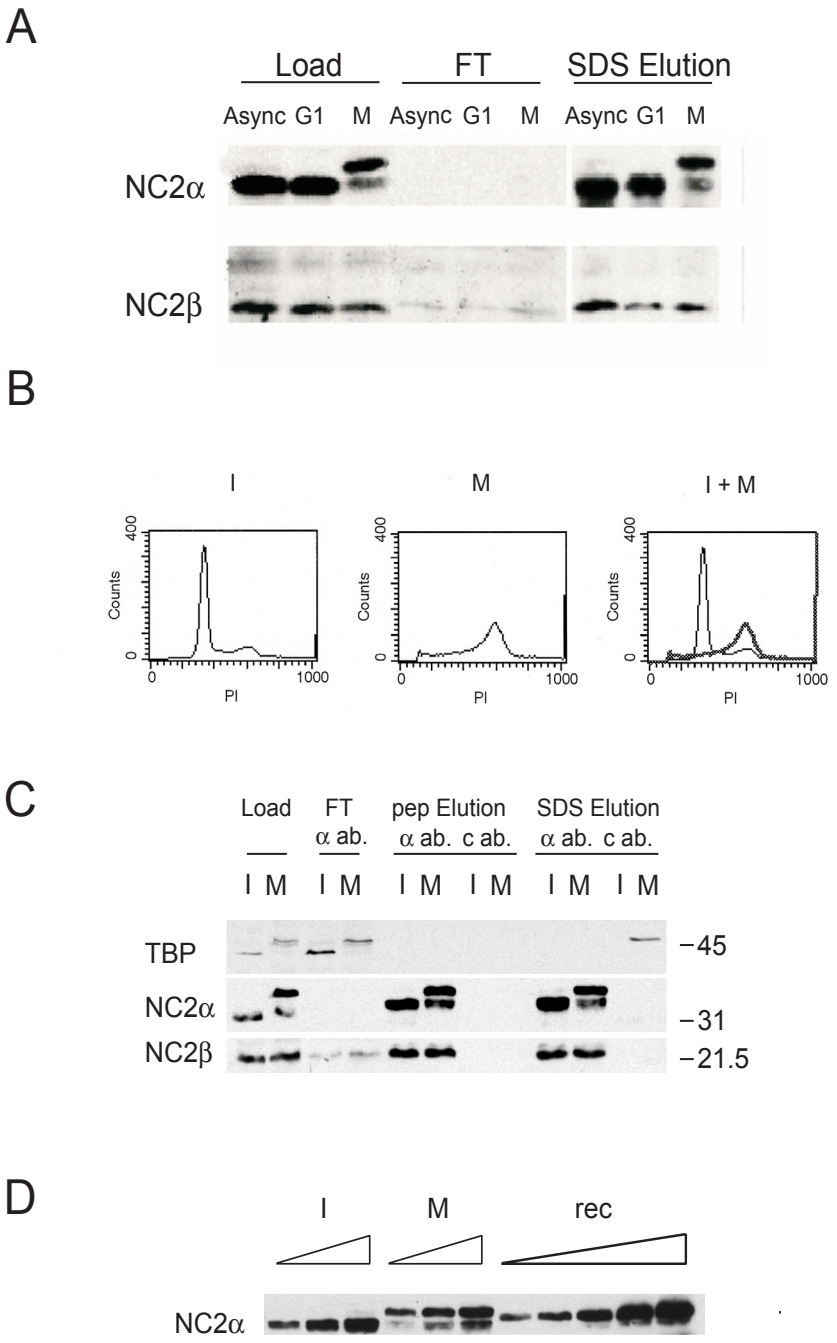


Figure 20. NC2α and NC2β remain associated following mitotic phosphorylation. (A) NC2α was immunopurified from asynchronous, G1 and M WCE. Immunoprecipitates were fractionated by SDS-PAGE, blotted to nitrocellulose membrane and NC2α and NC2β were detected with the specific antibodies. NC2β coimmunoprecipitated with NC2α in all the extracts. (B) Flow cytometry analysis of the DNA content (PI) of Interphase (I) and mitotic (M) blocked HeLa cells (see methods). (C) Control Western Blot of the immunoprecipitation of NC2 from I and M WCE. Proteins were eluted first with a peptide and then with SDS buffer. Immunoprecipitation with an isotype unrelated ab (CAD9) was used as a negative control. The membrane was probed with the antibodies as indicated on the figure. NC2α is immunoprecipitated only with the specific ab. NC2β coimmunoprecipitates with NC2α in both extracts. TBP does not coprecipitate with NC2, even in the M extracts. (D) Analysis by immunoblot of the immunopurified I and M NC2 compared to recombinant protein. The blot shows increasing amounts of NC2 proteins. 2, 4 and 8 μl of native NC2 were used; 10, 20, 40, 80 and 160 ng of recombinant NC2 protein were used. From the blot, the native I and M NC2 were estimated to be between 5-10 ng/μl. α ab = NC2α antibody; c ab = control antibody. FT = flow through.

2.8.2. The mitotic NC2 maintains the ability to bind to TBP-promoter complexes

EMSA was used to compare the binding of recombinant, G1 and M native NC2 populations to recombinant TBP and to DNA. Since G1 constitutes part of the interphase, these two phases can be considered equivalent in this contest. The DNA used for the assay was a 35bp [^{32}P] labeled oligonucleotide containing the TATA box of the Adenovirus Major Late Promoter (35-AdMLP). Protein-DNA complexes were resolved on non-denaturing polyacrylamide gel and visualized by autoradiography. The same amounts of active protein for recombinant and endogenous NC2 were compared for binding ability.

G1- and M-NC2 bind equivalently to TBP-DNA (Fig. 21a), suggesting that the extra charges acquired by phosphorylation during mitosis do not prevent NC2 from making stable contacts with TBP-DNA complex. This observation is surprising, because of the apparent subcellular relocation of NC2α to the cytoplasm during mitosis.

Interestingly, complex formation is neither increased nor reduced when the native protein is used instead of the recombinant one, suggesting that phosphorylations of native NC2 does not affect its binding to DNA.

2.9. Complex formation in a long DNA fragment: phosphorylation reduces affinity for DNA

To investigate the possibility that the mitotic hyperphosphorylation affects complex formation depending on the DNA, the binding ability of M-NC2α versus interphasic and recombinant NC2 was tested. EMSA experiments were performed keeping TBP constant, while NC2 was added in increasing amounts using different DNA templates.

In a first experiment we used a long DNA template containing as before the TATA AdML promoter (217 bp long). In contrast to the previous observations, the endogenous NC2 and M-NC2 proteins showed lower binding ability than the recombinant one (Fig. 21b). Compared to recombinant NC2, almost 3-times more native protein was necessary to bind the same fraction of DNA (140 fmol vs. 40 fmol, respectively; compare lanes 9 and 13 versus 5), indicating that phosphorylation reduces affinity.

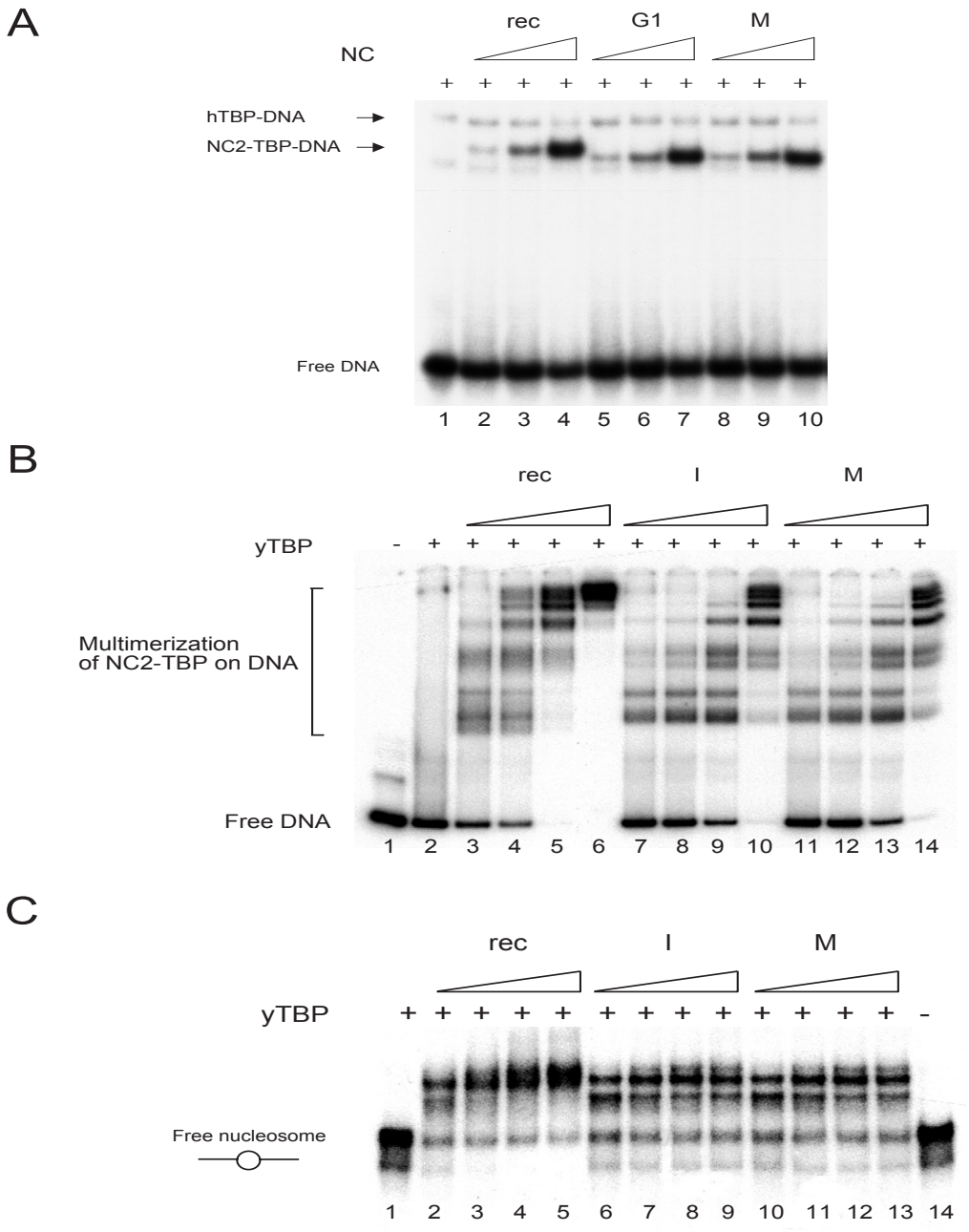


Figure 21. TBP-DNA binding ability of M-NC2 versus I- and reconbinant- NC2. between NC2 proteins. (A) Electro mobility shift assay (EMSA) performed using a DNA oligo of 35 bp containing the Adenovirus Major Late Promoter (35 bp AdML). Three different NC2 preparations at increasing amounts (20, 60, 180 fmol) were compared for binding ability to TBP-DNA complexes: recombinant NC2, G1 and M endogenous NC2. All lanes contained 38 ng of hTBP and 100 fmol of DNA. M NC2 shows the same complex formation ability of G1 and recombinant NC2. (B) EMSA with longer DNA fragments (217 bp) containing the Adenovirus Major Late Promoter derived from the pB2-MLP vector. Again, increasing concentrations of the three different NC2 preparations (30, 60, 120, 350 fmol) were compared for binding ability to TBP-DNA complexes. All lanes contain 100 fmol and 5 ng of yTBP (except the first line, where yTBP is absent). Both native proteins showed lower binding ability than the recombinant. Several bands are observed corresponding to different NC2-TBP-DNA complexes, depending on the number of NC2-TBP bound to the fragment. (C) EMSA was performed with proteins amounts described in B, but using as a template ca. 100 fmol of DNA, containing a nucleosome in the middle (see methods). Increasing amount of M NC2 (and I NC2) does not correspond to an augment in binding, in contrast to what observed for recombinant NC2.

2.10. Complex formation in a nucleosome template

We also tested binding to *in vitro* reconstituted nucleosome-DNA, which contained the nucleosome in the middle of the fragment. Figure 21c shows that binding of recombinant protein is proportional to its amount, whereas this positive correlation is not observed for the endogenous proteins, whose binding ability is constant.

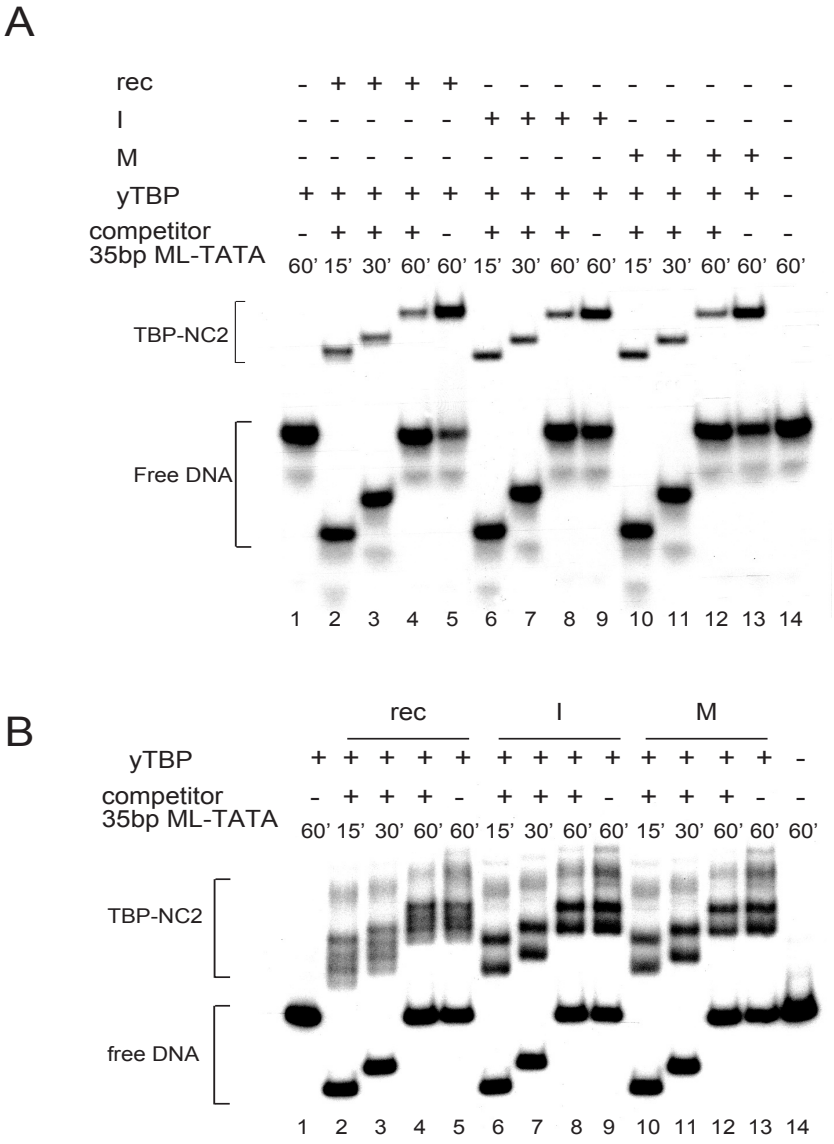


Figure 22. The mitotic hyperphosphorylation of NC2 does not affect stability of NC2-TBP-DNA complex. (A) Kinetic analysis of NC2-TBP dissociation. EMSA was performed with 350 fmol (25 ng) of yTBP, 10 fmol of recombinant NC2 and 20 fmol of labeled DNA oligo of 35 bp containing the Adenovirus Major Late Promoter (35 bp AdML). NC2, yTBP and DNA were pre-incubated for 30 min at 28 °C. Then, an excess (5000x) of cold competitor 35 bp Ad-ML promoter oligonucleotide was added to the reaction and incubated at 28 °C for 15 min (lanes 2, 6, 10), 30 min (lanes 3, 7, 11) and 60 min (lanes 4, 8, 12). As a control, samples were incubated for 16 min in absence of competitor DNA (lanes 5, 9, 13). After 1 h of incubation with competitor DNA, 40% of DNA is still bound, compared to the control, independently from the NC2 preparation. (B) EMSA was performed as described in panel A, but using as labeled DNA a long fragment (217 bp) derived from pB2-MLP vector (kindly provided by M. Timmers). After 1 h incubation with competitor DNA,

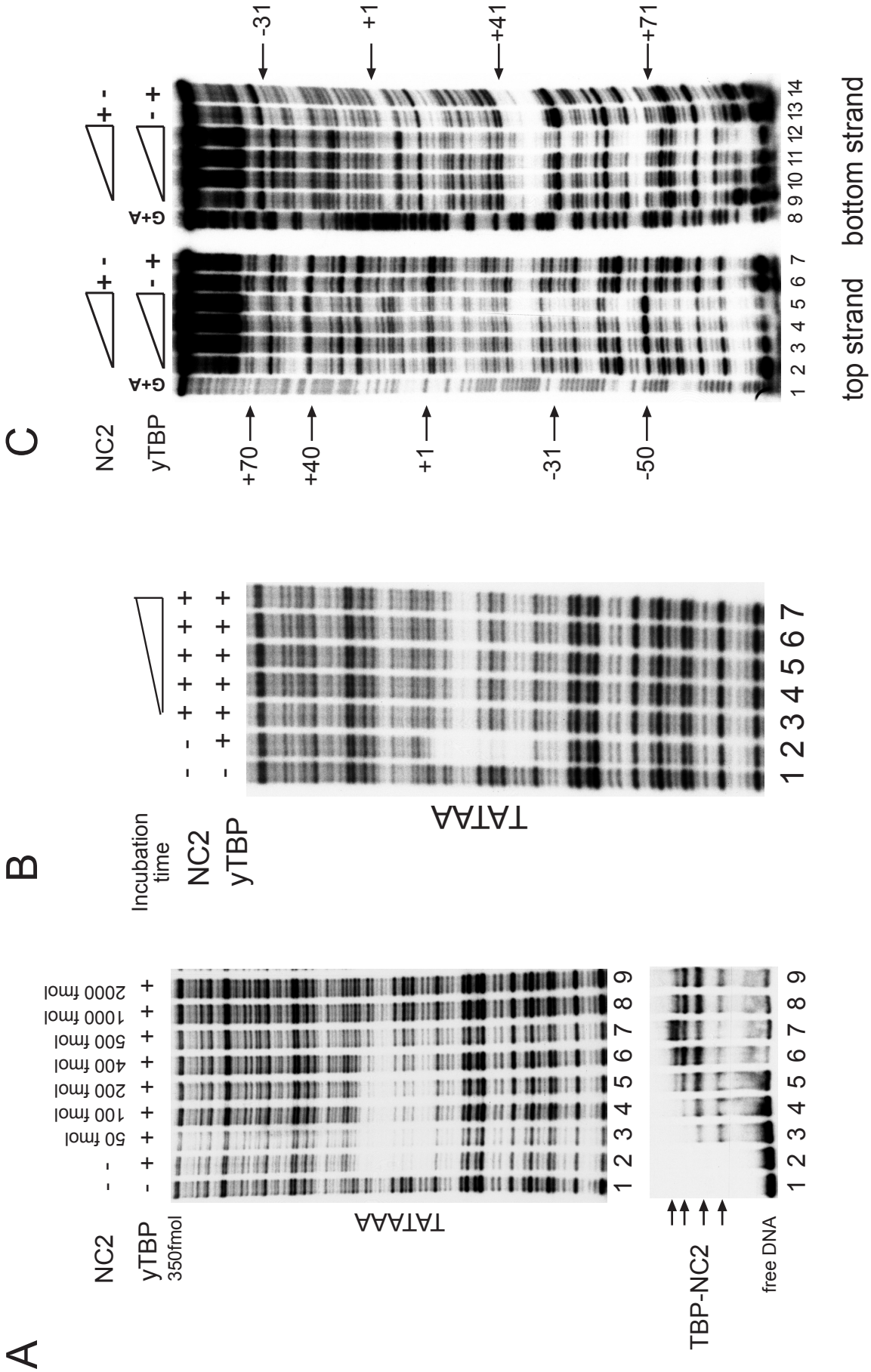
2.11. NC2-TBP-DNA complex is more stable in the long DNA, independently from the phosphorylation state

Next, we asked if the stability of the complex was affected by the mitotic phosphorylation. To test if NC2 phosphorylation can affect the dissociation rate and the stability of the complex, competition experiments with EMSA were performed. Recombinant and native NC2 were incubated with hot DNA and TBP for 30', and then an excess (5000x) of cold competitor 35bp AdML was added. After incubation for increasing time points, the samples were loaded on a gel. Two different labeled DNA templates were used: a short 35bp and a longer AdML promoter fragment. With the short DNA, the half-life of the complex was estimated to be around 30' (Fig. 22a). In contrast, when the template was the long DNA fragment, ca.85% of the complex was still bound even after 1h (Fig. 22bb). Thus, in the long DNA the complex is more stable than with short DNA. Recombinant and native NC2 behaved identically, suggesting that phosphorylation does not influence dissociation of the trimeric complex.

3. The role of phosphorylation of NC2 on complex mobilization

3.1. NC2 mobilizes TBP on DNA

Previous work showed that recombinant NC2 and TBP protects the TATA-box of a HIV-1 promoter DNA from DNaseI digestion (Goppelt et al., 1996; Kim et al., 1996). The footprint pattern in the presence and absence of NC2 is very similar, with minor differences seen at the border of the protected region (Goppelt). Similar effects have been described in the case of the AdML-Promoter (J.Kim et al., 1996).
Yet, the role of phosphorylation was not studied in this contest.
On the coding strand of an AdML-Promoter DNA, TBP protect a window surrounding the TATA box (Fig. 23a lane 1 versus 2). This window was gradually converted to an unprotected region upon addition of increasing amount of NC2 (lane 2 versus 8). The EMSA experiment, made in parallel with aliquots of the samples not digested with DNaseI, argues against a dissociation of TBP-NC2 from the DNA (Fig. 23a bottom panel). In fact, bands corresponding to the trimeric complex DNA-TBP-NC2 are always visible. These bands increase gradually up to lane 7 and then decrease



See next page for the legend.

(compare lane 8,9 versus 6). This could be explained supposing that when present in excess, NC2 complexes with itself in solution, resulting in an NC2-NC2 tetramer that sequesters some NC2 from binding to the DNA.
The disappearance of the protected region, and the concomitant presence of higher order complexes on the DNA, strongly indicates that NC2 moves TBP from the TATA box, but does not dissociate from the DNA. Moreover, the absence of other protected regions suggests that the mobilized TBP-NC2 does not have preferences for other sites.

To further investigate this phenomenon, a time course experiment was performed: TBP was first incubated with the DNA at 28°C and then the reaction was incubated at 4°C together with NC2 at different time points (Fig. 23b). The appearance of hypersensitive sites upon addition of NC2 is observed already after 30sec, indicating that TBP mobilization is fast.

3.2. At 4 °C NC2 confers to TBP a certain preference for binding

In a different footprint experiment, all the incubations were done at 4°C and both TBP and NC2 were titrated (Fig. 23c). At the lowest NC2-TBP concentrations, the pattern of hypersensitive sites is almost identical to the control lane. At very high NC2-TBP concentrations, several protection windows appeared, in a manner dependent on both proteins (lanes 5 versus 6, 7 and 12 versus 13,14). Thus, while footprints performed at room temperature show that NC2 mobilizes TBP from TATA box, low temperatures confer to TBP preference for certain sites.

Figure 23 (previous page). Mobilization of TBP by NC2. DNAseI footprinting analysis of NC2 and TBP. (A) Increasing amounts (as indicated, in fmol) of recombinant NC2 were added to 350 fmol of yTBP that was preincubated for 30 min at 28 °C with approximatively 100 fmol of a 217 bp Ad-ML fragment, labeled by PCR using radioactively labeled primers (top panel; see methods). In parallel, EMSA analysis was performed, where 25% of the reactions were loaded on native gels before addition of DNAseI (bottom panel). Increasing amount of NC2 correspond to disappearance of the protected TATA region. Although mobilized, NC2-TBP complexes are still bound to DNA, as shown in EMSA (bottom panel). (B) Time course of TBP mobilization from TATA. 350 fmol TBP were incubated with the DNA, and then 400 fmol of NC2 were added to the reaction for increasing time points (30 sec and 3, 6, 15, 40 min). Mobilization of TBP by NC2 is observed already 30 sec after NC2 addition. (C) Footprinting analysis of NC2-TBP at 4 °C. Ad-ML promoter fragment (pB2 MLP-Small) was preincubated with TBP (60, 140, 350 and 700 fmol) for 30 min at 28 °C, followed by addition of NC2 (125, 250, 500 and 1000 fmol) and subsequent incubation for 30 min on ice and DNAseI digest (30 mg for 30 sec). Only at high NC2-TBP concentrations, some protection windows appear.

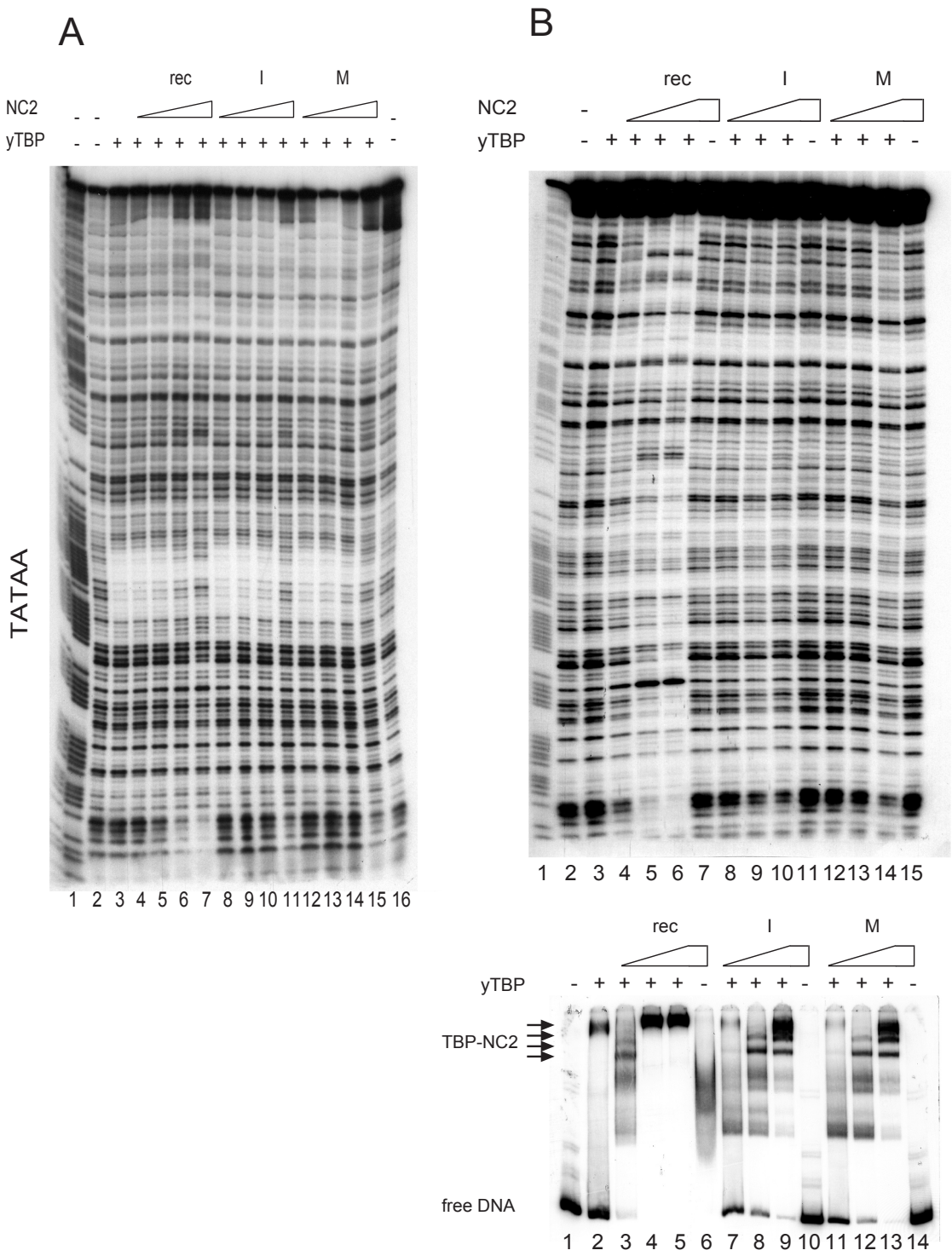


Figure 24. The mitotic hyperphosphorylated NC2 mobilizes TBP. (A) Footprinting analysis was performed with the same conditions described in Figure 23, using increasing amount of recombinant and endogenous NC2 from I and M blocked cells (60, 120, 350 fmol). Both recombinant and native NC2 mobilize TBP on the DNA. (B) Footprinting analysis was performed reproducing the experiment in Fig. 23b, but using recombinant and endogenous NC2 (250, 500, 1000 fmol). At high NC2 concentrations, the M NC2 and the I NC2 still do not produce the protected regions observed for the recombinant NC2-TBP complex.

3.3. Recombinant and native NC2/M-NC2 behave differently in footprint

Next, we asked if the mitotically hyperphosphorylated NC2 and phosphorylation in general influenced TBP mobilization by NC2.
In a footprint experiment done at room temperature, the addition of increasing amount of recombinant NC2 to TBP-DNA causes the disappearance of the protected window corresponding to TATA. Equal active protein concentrations of recombinant and native NC2 were compared in the same assay: at the highest concentrations, both recombinant and native NC2 cause the disappearance of the TBP-protected window (Fig. 24a). This was in contrast to the EMSA performed in parallel to the footprint: three times more native protein is necessary to reach the same binding observed for recombinant protein (Fig. 21b). Again, the two native proteins had the same behavior.

When the foortprint was performed at 4°C, a notable effect of phosphorylation became evident (Fig. 24b). As seen before in the footprint at 4°C, in the presence of recombinant NC2, TBP-NC2 shows a preference for certain sites and makes several windows on DNA. In contrast, the windows are only weakly detectable with the endogenous protein. Also in this case, the EMSA done in parallel to the footprint confirmed the different DNA binding ability of TBP-NC2 containing the native protein (Fig. 24b bottom panel). No significant difference was detected between I-NC2 and M-NC2.

4. Identification of NC2 isoforms

Post-translational modifications (PTMs) can change the protein properties by proteolic cleavage or by addition of a modifying group to one or more amino acids. In particular, PTMs can determine the protein activity state, localization, turnover, and interactions with other proteins.
As shown before, there is evidence that both NC2 subunits are phosphorylated *in vivo* and *in vitro*, in agreement with previous studies that did not characterize in full detail the modification sites (Inostroza et al. 1992; Goppelt et al. 1996; Creton et al. 2002).
To increase our knowledge on this process, we performed two-dimensional gel electrophoresis (2D gel) to estimate the number of NC2 phosphorylation events. 2D gel electrophoresis separates protein populations on the basis of (i) charge

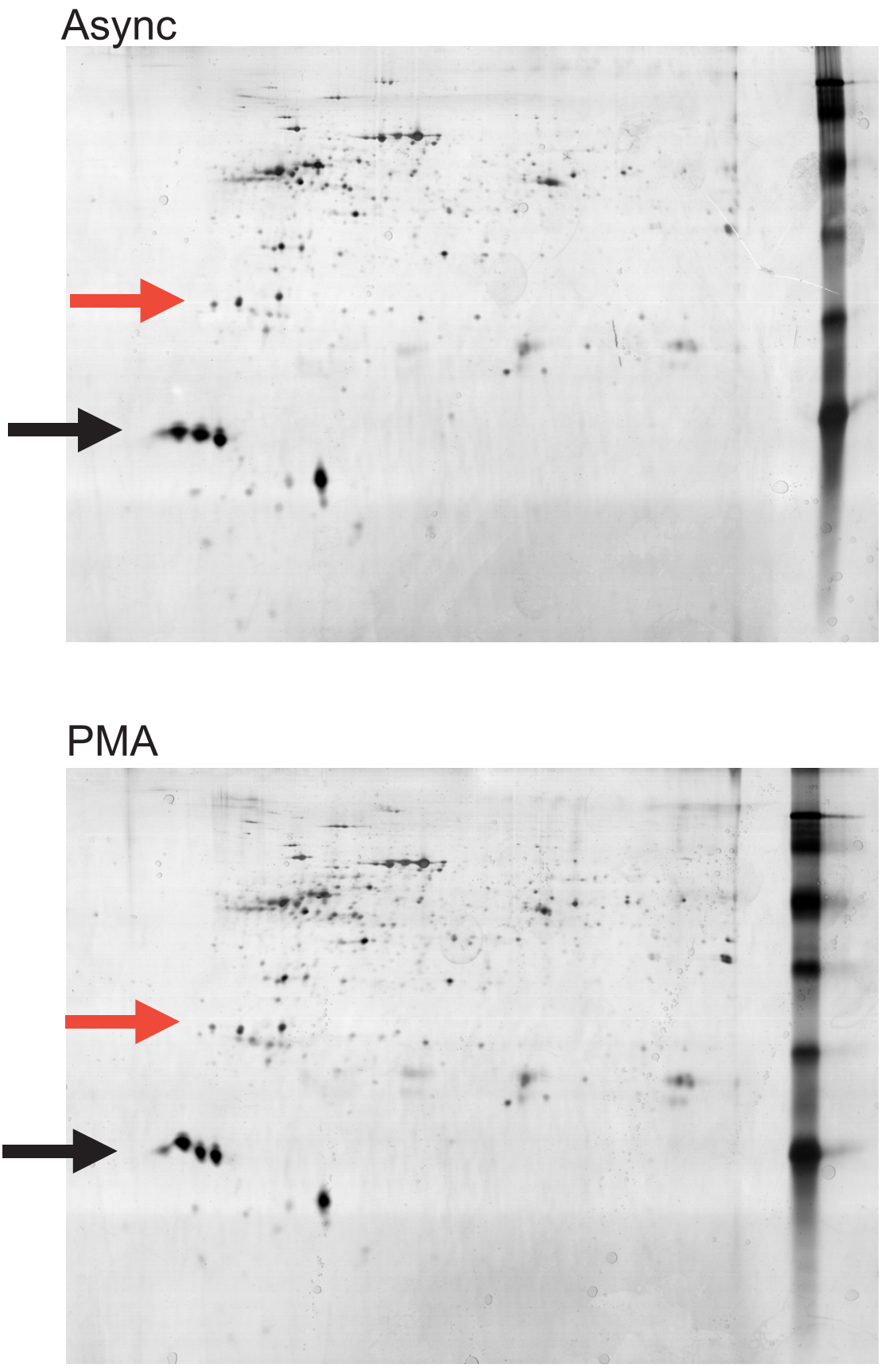
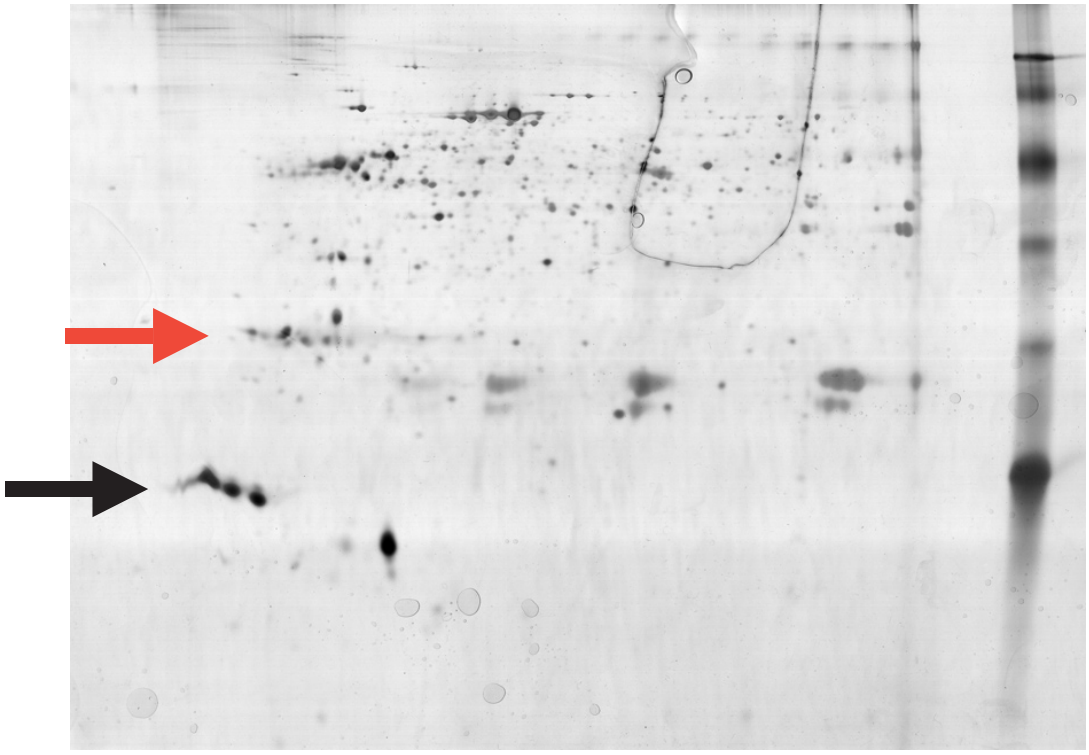


Figure 25. Continues in following pages.

M



c Async

Figure 25. Continues in following page.

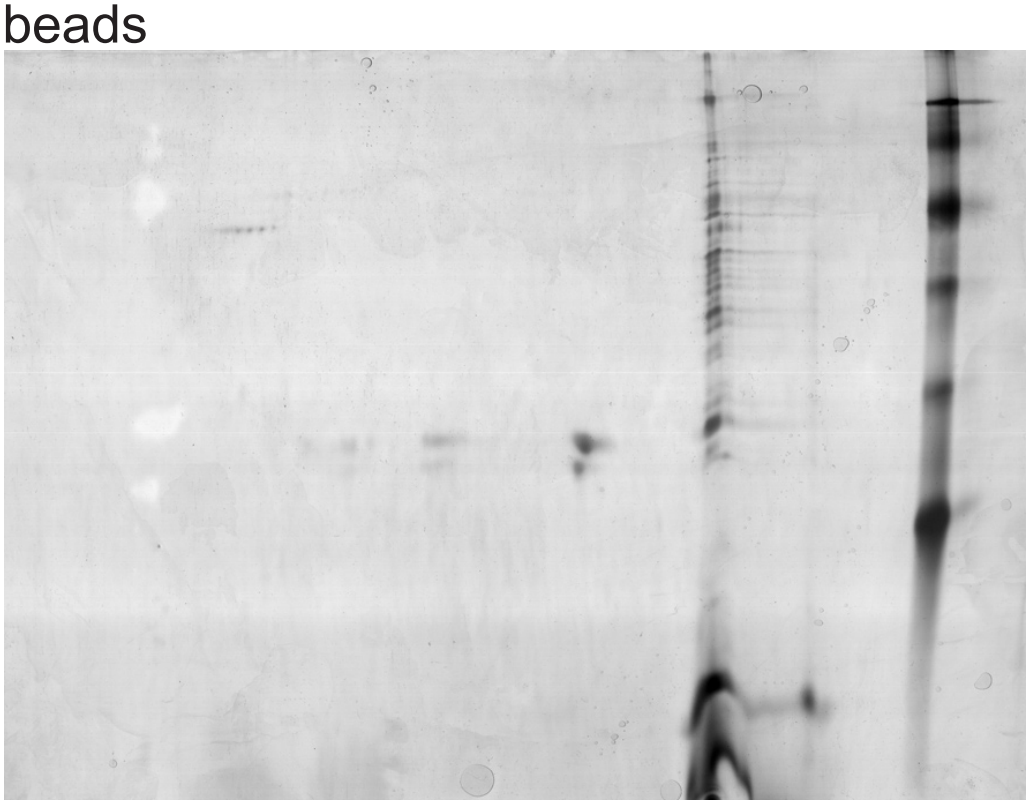


Figure 25 (continues form previous pages). Both NC2α and NC2β have several isoforms. 2D electrophoresis analysis of NC2. Each gel was loaded with NC2 immunopurified from WCE of three different populations: asynchronous (async), asynchronous + PMA stimulation (for 30 min before harvesting; async+PMA), and mitotic blocked (M) Jurkat cells. As a control, the asynchronous WCE were also incubated with a column with bound an antibody of the same isotype than the NC2α ab that does not recognize any protein in the WCE (c async). Proteins were resolved using isoelectric focusing (pH 4-7) and 13% SDS-PAGE. The migration of molecular mass standards (kD) is indicated on the right of the panel. Proteins were visualized by silver stain. NC2β spots are indicated by black arrows, the NC2α ones by red arrows.

and (ii) molecular weight. If a protein is phosphorylated, the resulting change in its charge usually produces a horizontal trail of protein spots.

The aim was to study:

(i) the normal *in vivo* phosphorylation state of NC2; (ii) the hyperphosphorylation characteristic of mitosis; and (iii) the phosphorylation events following cellular signals, since both proteins (subunits alfa and beta) contain potential phosphorylation sites for the protein kinase C (PKC), a kinase activated upon stimulation of the cells with phorbol myristate acetate (PMA). Among the potential PKC sites, NC2β contains two particularly interesting ones (serines 105, 106), located between the DNA and the TBP interaction domains. Phosphorylation at these sites could produce repulsion with the DNA backbone and destabilize the NC2-TBP-DNA complex in a signal dependent manner.

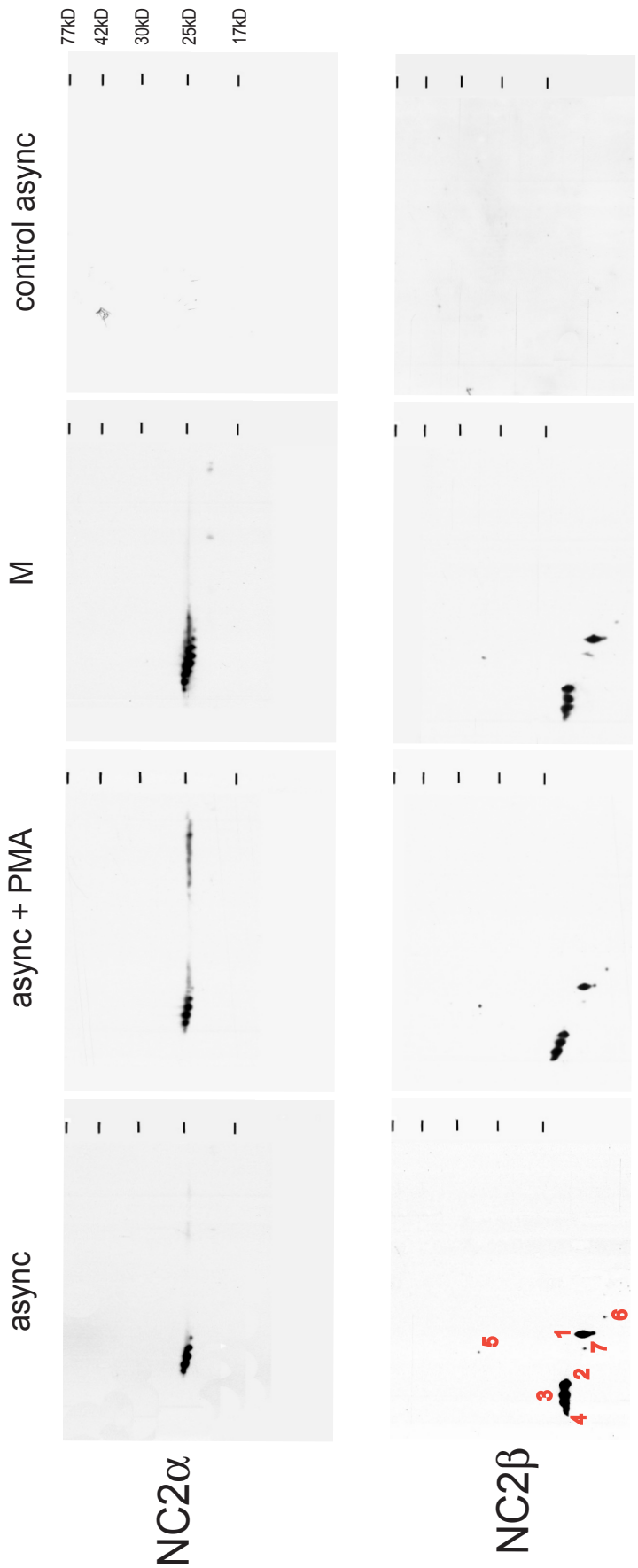
Since direct analysis of modifications requires isolation of a large amount of correctly processed protein, we used Jurkat cells, which could be grown in large numbers. Large scale preparations of Jurkat WCEs were prepared from asynchronous cells (stimulated or not with PMA; async-PMA and async, respecvively), and M blocked cells. Aliquots of these cultures were checked in flow cytometry analysis to record the status of the cells.

2D gels can be performed with cell lysates, but it is often advantageous to reduce the complexity of the mixture and increase the amount of the protein of interest before loading it onto the gel. Therefore, NC2 was purified from the extracts via immunoprecipitation with an antibody directed against the NC2α subunit (4G7). As a control, extracts were incubated with an isotype ab before the NC2α specific ab. Then, the bead coupled with the isotype and the one with the specific antibody were washed and NC2 was eluted with urea buffer compatible with 2D gel system. Small aliquots of the elutions were checked in WB to confirm that NC2 had been depleted from the extracts and eluted from the specific antibody but not from the isotype one. To control the quality of the immunoprecipitation, an aliquot of the proteins contained in the elutions was separated by SDS-PAGE and stained with silver stain. In general, the purity of the elution was not high, probably due to the low stringent conditions of the washes (150 mM KCl).

The immunoprecipitations were finally analyzed by 2D gel (Fig. 25). Since the purity of the samples was not high, three 2D gels were run in parallel from the same probe, one containing 80% and the other two 10% each of the total material. While the first gel was used for the silver staining, the others were blotted in a PVDV membrane and stained with the NC2α and NC2β antibodies, in order to recognize the specific NC2 spots. The immunoprecipitates were resolved in the first dimension by isoelectric focusing in a pH range from 4 to 7, and in the second dimension by SDS-PAGE (T=13%, C=3%). 2D gels were made from the three specific probes: async, async-PMA, M elutions. In addition, we ran 2D control gels also of (i) the elution of the async WCE immunoprecipitated with the isotype antibody; (ii) the elution of the beads coupled with the antibody (light chain); (iii) recombinant NC2 protein. Comparing the 2D silver stain with the corresponding 2D WB allowed distinction the NC2α and NC2β specific spots from the other proteins.

4.1. NC2α and NC2β have different isoforms

Both subunits showed several isoforms. NC2α resolved as one molecular mass species, but at least four distinct isoelectric points were recognizable, in both the



See next page for the legend.

asynchronous and the PMA stimulated cells (Fig. 26). Moreover, an additional distinct isoelectric point was observed in the M cells.

These data clearly demonstrate the presence of several forms of NC2α, and strongly confirm the findings of the previous experiments, pointing to the existence of a mitosis specific form of NC2α.

Surprisingly, NC2β resolved as four molecular mass species and at least seven different isoelectric points, four of which were visible as clear big spots (n.1, 2, 3 and 4 in Fig. 26). Presumably, the NC2β observed as a single band in a standard WB corresponds to the series of the three big spots n.2, 3, and 4: these are likely three differentially phosphorylated forms of the protein, with small isoelectric shift. The silver stain suggests that spot n.4 is as abundant as the three previous ones, with the difference in size pointing to a deletion form of NC2β. Interestingly, this form is not observed in normal 1D gel/WB with antibody stain, likely because, in comparison, the 2D gel/WB have a very high protein amount. A new potential high mobility NC2β form had already been observed marking NC2 with radioactivity, a very strong label compared to antibodies (compare with Fig. 18d).

The other NC2β spots (n.5, 6, 7) have not been observed before, but they are present in each of the three preparations, arguing against an artefact. The spot n.7 has same molecular weight but different PI than n.1, suggesting that it could be the corresponding phosphorylated form. The spot n.6 could correspond to a form even shorter than the spot n.1. Finally, the spot n.5 has a size that could correspond to a NC2β dimer.

PMA stimulation does not seem to change the phosphorylation state of NC2, since the same pattern of spots is observed in the async and PMA gels/WBs for both NC2α and NC2β. This does not necessary exclude that Ser 105-106 are phosphorylated, since they could be modified by a constitutively active kinase instead of PKC or be rather unstable or underrepresented.

Figure 26 (previous page). 2D Western Blot analysis of NC2α (upper panel) and NC2β (bottom panel). Three gels were run in parallel from each of the three samples (async, async+PMA and M); two of them were transferred to a PVDV membrane and immunostained with either NC2α or NC2β antibodies. The migration of molecular mass standards (kD) is indicated on the right of the panel. NC2α resolved as one molecular mass species, but at least four distinct isoelectric points were recognizable, in both the async and the async+PMA. The mitotic extract clearly shows an additional distinct isoelectric point. Seven different points were recognized by NC2β ab (numbered 1-7): spots 2-4 correspond to the band normally observed in WB.

4.2. Mapping of the phosphorylation sites of NC2β

The silver stain gels were further processed for the matrix-assisted laser desorption/ ionization (MALDI)-TOF analysis of the spots, to map possible protein modifications. A theoretical approach using a motif-finder database (Expasy) identified several putative phosphorylation sites in both NC2α and NC2β (Fig. 2b). The analysis of the peptides obtained by in silico digestion of NC2α and NC2β showed that none of the commonly used chemicals or proteases gives a suitable pattern of peptides for NC2α, since none of the digestions was able to cut the C-terminus, which contains most of the phosphorylation sites. On the contrary, NC2β digestion with Glu C resulted in a set of peptides with suitable size and covering all the

Table 1. Fingerprinting analysis of the NC2β isoforms. Spots 1-5 identified as NC2β in the 2D WB (Fig. 27, bottom panel) were cut from one gel (async + PMA), digested with Glu C and analyzed my mass spectrometry. All five spots were confirmed to be NC2β. Only few of the expected peptides were identified by MALDI-TOF. Two peptides were phosphorylated at one site. Mass is expressed in Dalton. Position is relative to NC2β sequence. The list reports the peptides expected after digest of NC2β with GluC; bold-underlined residues are the potential phosphorylation sites. Numbers 1-5 correspond to the spots of the gel async + PMA in Figure 27, bottom panel. – = undetected peptide. UNMODIFIED = peptide detected without modification. P = peptide detected as phosphorylated at one site. OH = peptide detected as unphosphorylated.

mass	position	peptide sequence	1	2	3	4	5
768.2828	1-8	MASS**S**GND	OH	P	P	OH/P	OH/P
1597.9458	11-24	LTIPRAAINKMIKE	NOT PHOSPHORYLATED				
1098.5902	25-34	TLPNVRVAND	-	-	-	-	-
880.3903	38-45	LVVNCCTE	-	-	-	-	-
945.5040	46-53	FIHLI**S**SE	NOT PHOSPHORYLATED				
693.3236	57-62	ICNK**S**E	-	-	-	-	-
802.4669	63-69	KKTI**S**PE	-	-	-	-	PP?
809.4515	70-76	HVIQALE	UNMODIFIED				
1059.4993	77-86	**S**LGFGSYISE	P	P	P	P	P
1846.0803	94-109	CKTVALKRRKA**SS**RLE	-	-	-	-	-
900.4309	110-117	NLGIPEEE	UNMODIFIED				
914.5054	118-124	LLRQQQE	+	+	+	+	-!
1289.6960	125-135	LFAKARQQQAE	UNMODIFIED				
588.2987	136-140	LAQQE	-	-	-	-	-
3205.4450	141-171	WLQMQQAAQQAQLAAASA**S**A SNQAG**SS**QDEE	-	-	-	-	-

potential phosphorylation sites (Tab. 1). Therefore, five of the spots identified in WB as NC2β were cut from the gel, digested with the Glu C and analyzed by MALDI-TOF (spots n.1-5 in Fig. 26). The experimentally determined peptide masses were matched against those expected from the protein sequence. Only part of the expected peptides was detected, and the peptide containing the serines 105-106 was not among them. Most of the peptides were unmodified, but few were found carrying a phosphorylated residue (Tab. 1). The most interesting findings are that (i) the peptide 1-8 contains 1 phosphorylated serine (out of the three present in this stretch), probably serine 5, as it was also identified as potential CKII phosphorylation site; (ii) neither of the 2 serines of the peptide 46-53 is phosphorylated. (iii) Peptide 63-69 might have two phosphorylated sites; (iv) the peptide 77-86 contains 1 phosphorylated serine (out of the three present in this stretch), and serine 77 is the best candidate because identified as a potential phosphorylation site.

The only differences observed among the five spots are: (i) the first peptide has one serine that is phosphorylated in all the five spots except the n.1; (ii) the mass spectrum of spot 5 was identical to those of the other spots, although peptide

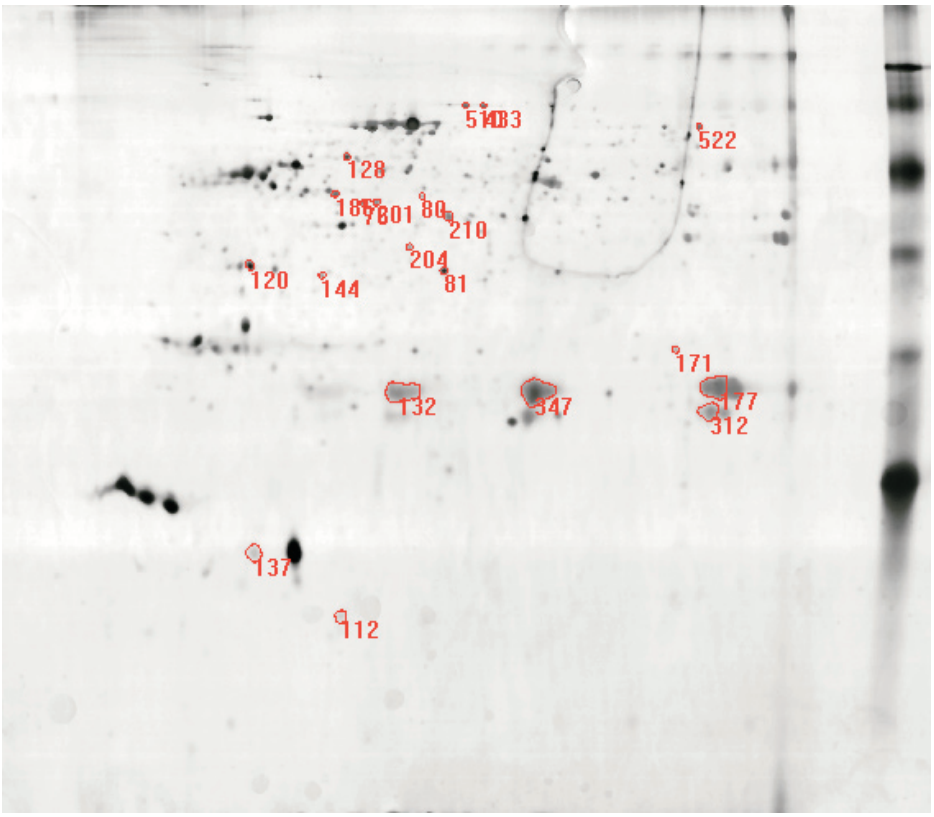


Figure 27. Identification of proteins coimmunoprecipitating with NC2. (A) Computer densitometry analysis. The digitalized images of 2D gels made from three samples (async, async+PMA and M; see Fig. 25) were compared with the control gel (c Async). The proteins coimmunoprecipitating exclusively with NC2 were recognized after subtracting the spots present in the control gel. These proteins correspond to the numbered spots in the gel, and are listed in Table 2.

Intensity of the significant spots				
Spot ID	Control	Mitotic	PMA	log
76	9.9	51.7	38.5	28.5
80	7.4	23.4	13.1	13.8
81	11.9	100.8	112.5	114.8
112	N.A.	54.8	51.9	150.3
120	N.A.	153.9	242.1	180.6
128	N.A.	70.3	85.0	68.2
132	N.A.	665.6	162.5	132.2
137	N.A.	146.7	78.4	163.5
144	N.A.	38.0	42.5	44.0
171	N.A.	32.3	42.8	48.2
177	N.A.	873.1	440.2	238.4
186	N.A.	72.9	77.1	60.9
201	N.A.	51.3	38.9	28.0
204	N.A.	35.6	29.1	25.9
210	N.A.	114.6	81.1	75.3
312	N.A.	333.1	102.9	N.A.
347	N.A.	1086.1	442.8	279.9
433	N.A.	42.6	N.A.	N.A.
510	N.A.	46.6	N.A.	N.A.
522	N.A.	40.9	N.A.	N.A.

Table 2.

118-124 was not identified. Beside that, no significant difference was observed among these five isoforms, suggesting that the observed isoform heterogeneity must reside in the unidentified peptides.

5. Search for potential NC2 interactors

Next, we wanted to determine the identity of the proteins coeluted with NC2α. A computer densitometry analysis was performed, in which the intensity of the spots present in the async, PMA, and M gels was compared to those in the control one (made with async extracts incubated and eluted with the isotype antibody). The analysis revealed 20 spots present exclusively in the NC2α IPs (Fig. 27 and Table 2). Of those, 4 belonged to the light chain of the antibody and other two were NC2β isoforms (the spots 6 and 7 identified in the 2D WB). The remaining spots were cut and processed for the MALDI-TOF analysis. All the spots were identified, and the list of the corresponding proteins is reported in Table 3. None of the identified protein seems related to the transcription process and thus to NC2. Interestingly, four spots are specific for mitosis, suggesting interactors specific for the mitotic hyperphosphorylated form of NC2α.

n. spot	MW	Proteins
80	62	Hypothetical protein DKFZp586G0322.1 (fragment)
81	34	Ribosomal protein P0
120	35	Dermal papilla derived protein 6 (DERP6)
128	61	Chaperonin (HSP60)
144	82	Unnamed protein product (Homo sapiens)
171	30	TRAF4 associated factor 1
186	64	HSPA8 protein
210	71	BiP protein
433	64	Chain B, crystal structure of the Ku heterodimer
510	71	Nuclear factor IV
522	70	Thyroid autoantigen 70kD (Ku antigen)

Table 3. Peptide mass fingerprinting of the spots coimmunoprecipitating with NC2. The table list the spots identified by MALDI-TOF proteins.

In another experiment NC2 was immunopurified from HeLa nuclear extracts. The proteins coeluted with NC2 were separated on SDS-gel and analyzed by mass spectrometry. The conditions for the IP used in this experiment were slightly different from the previous one. The extracts were first incubated with beads coupled to an isotype antibody (HA) and next with beads coupled to the NC2α monoclonal antibody, but then only the beads binding the specific antibody were washed and eluted. Moreover, NC2 was eluted from the antibody with a peptide matching the epitope of the ab. Thus, this IP was eluted more specifically, but on the other hand a control isotype was missing. Although the preparation checked in SDS-PAGE silver stained looked very cleaned, the mass spectrometry analysis identified around 30 different proteins coeluting with NC2. Only few proteins are related to transcription, like the TAFII68, Bcl3 and p105. An aliquot of the same NC2 preparation used for the MS analysis was used for checking in WB if these proteins were also recognized by the specific antibodies. Bcl3 and p105 were in fact confirmed, whereas TAFII68 was not reconfirmed. However, since the control IP with the isotype antibody was missing, these proteins could be NC2 interactors as well as contaminants. A single protein was identified in both the experiments, BiP, also known as Heat-shock 70kD protein-5, or glucose-regulated protein, 78kD.
Further studies will be necessary.

III. DISCUSSION

1. Localization of NC2

1.1. Identification of nuclear localization signals (NLS) in both NC2α and NC2β

In this study we provided evidence for the presence of nuclear localization signals in both NC2α and NC2β.

NC2α contains a bipartite N-terminal NLS (4-KKKKYNARFPPARIKKI-20), which includes two highly positively charged motifs spaced by 8 amino acids. It is noteworthy that the acidic residues of the second motif (R16, K18 and K19) are involved in DNA contacts, and also in protein dimerization. These amino acids are conserved in all eukaryotes, while the first acidic motif (K4-7) is conserved in metazoans, but not in yeast (see Fig. 3). Unexpectedly, mutation of the second NLS (R16A/K18A/K19A) affected NC2α import less than mutation in the first one (K4A/K5A/K6A/K7A). Moreover, both NC2 subunits were partially retained in the cytoplasm when the first was mutated, while mutations in the second NC2α NLS resulted in a nuclear distribution of the dimer comparable to the control. This finding indicates that the first motif is not only the strongest import signal for the single subunit, but is also necessary for the import of the dimer. In contrast, the second stretch participates only in the import of the single subunit.
In general, the position of an NLS in the transported protein is important for signaling function (Roberts et al. 1987; Nelson and Silver 1989). For example, insertion of the NLS of the SV40 T antigen in the buried hydrophobic domain of the pyruvate kinase, produces a non-nuclear protein (Roberts et al. 1987), indicating that the NLS must be exposed on the surface of the protein to interact with components of the import machinery. Since the crystal structure of NC2 in a complex with TBP-DNA has been resolved, it is possible to predict if the sequences encompassing the NLS are exposed. Interestingly, the first part of the NC2α NLS is immediately adjacent to the histone fold domain (HFD), which is responsible for dimerization of the two subunits, whereas the second motif is inside it (Kamada et al. 2001). Therefore, the second motif is not exposed when the two NC2 subunits dimerize,

explaining why it may play a role only in the transport of NC2α alone. Moreover, the spacer between the two motifs contains two prolines residues that introduce a bend immediately before the first helix of the HFD and cause the first 10 amino acids to stick out from the histone fold, facilitating the exposure of the NLS.

Immunofluorescence analysis of successive deletion constructs of the NC2β subunit located an active NLS in the sequence between amino acids 80-112. The putative NLS corresponds to a single stretch of positive amino acids (100-KRRK-103). Mutation of the two central positive residues of this sequence (R101A/R102A) had a minor effect on import of NC2β alone, but it did retain some NC2 dimer in the cytoplasm. NC2β NLS is located at the end of helix4, which is involved in DNA binding, but not in intermolecular interactions. Thus, this sequence is likely to be exposed to the surface of the protein also in the dimer.

To support our experimental results, we used a protein-sequence analysis program named PSORT (Nakai and Kanehisa 1992), which identifies NLS based on the presence of a certain number of basic amino acids (Hicks and Raikhel 1995). Analysis of NC2 sequences with PSORT recognized our potential nuclear import sequences as the only NLS in both subunits. Interestingly, the NLS identified in NC2 do not match any of the 91 experimentally verified NLS found in the literature and used to construct the PredictNLS prediction server (Cokol et al. 2000). Hence, the NC2 NLS could be different and thus be important for NLS identification in other proteins.

The finding that both NC2 subunits carry a NLS indicates that NC2 is imported in the nucleus via the classical import mechanism. This is based on recognition of the cargo by the heterodimeric importin receptor, which consists of the importin-α and importin-β subunits (Gorlich 1997). Importin-α binds directly to the NLS, whereas Importin-β targets the import complex to nuclear pores. To investigate this hypothesis, we tested the ability of the importin-α receptor to recognize NC2, incubating the GST-importin-α bound to a column with some recombinant and endogenous immunopurified NC2. Interestingly, preliminary data showed that both subunits, from either recombinant or endogenous NC2, were pulled down (data not shown). Also the mitotic hyperphosphorylated form of NC2α was recognized from the importin-α, but it was not possible to establish if the two forms were recognized with the same strength.

Yeast NC2α contains only the C-terminal motif of the NLS, which in human cells is effective in the import of the single subunit but not of the dimer. Moreover, yeast NC2β does not have the NLS identified in human and conserved in most of metazoans, nor does it contain other positively charged stretch that could substitute for it. This suggests that yeast NC2 may enter via diffusion or has a different mechanism of import than metazoans, perhaps via co-transport with other nuclear proteins.

1.2. Both the NC2α and NC2β NLS overlap with DNA-binding regions

Positively charged amino acids are important not only for the binding of a cargo to the import machinery, but also for DNA interaction. The observation that DNA-binding region and NLS-function overlap was initially made by LaCasse and Lefebvre (LaCasse and Lefebvre 1995). Later, a more extensive analysis of the proteins whose NLS and DNA-binding region were known, revealed that the two motifs overlapped for 90% of the proteins (Cokol et al. 2000). The colocalization of NLS and DNA binding regions might be related to the evolution of the eukaryotic system from the prokaryotic one. With the development of a nuclear membrane, which separates cytoplasm from nucleoplasm, the cells had to adopt a system to concentrate the nuclear proteins in this compartment. Since the DNA binding region is an exclusive and common motif among nuclear proteins, it is likely that evolution favored this motif as target of the import machinery, thus concentrating two functions in a single sequence. Consistent with this hypothesis, the NLS of NC2β and the 2nd acidic stretch of the NLS of NC2α coincide with DNA-binding regions. The establishment of an additional NLS in NC2α may be to promote efficient import of the dimer.

1.3. NC2β contains a potential nuclear export signal (NES)

Nucleo-cytoplasmic shuttling of transcription factors is an active process that relies on the recognition of their nuclear localization and/or export signals (NLS and NESs, respectively) by proteins of the nuclear import and export machinery (Hood and Silver 1999). Protein export from the nucleus is often mediated by a Leucine-rich Nuclear Export Signal (NES). Recently, a database for NESs has been created, which collects experimentally validated Leucine-rich NESs (la Cour et al. 2004). Submission of NC2 sequences to this database indicated that NC2 does not contain a known NES. However, a manual examination for the generally accepted

NES consensus L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI] (Bogerd et al. 1996), revealed a similar sequence in NC2β. This sequence is localized between the amino acids 99-LKRRKASSRLENLGI-113 and matches the C-terminal part of the consensus sequence (LENLGI = [LIVFM]-x(2,3)-L-x-[LI]). Many of the identified Leucine-rich NESs deviate significantly from the generally accepted loose consensus, although there are some preferences. A mutational study of the NES of the PKI protein indicated that the leucines in the C-terminal end of the signal are more important for function than the N-terminal ones (Wen et al. 1995), as confirmed from the high conservation of the C-terminal hydrophobic residues within a set of experimentally characterized NESs (la Cour et al. 2003). Consistent with that, the C-terminal -LENLGI- motif of the hypotetical NC2β NES matches the consensus NES, and these amino acids are conserved from *C. elegans* to *H. sapiens*.

In contrast, the first part of the potential NC2β NES diverges from the consensus sequence [L-x(2,3)]: after the first lysine, NC2β contains the NLS plus two serines (-LKRRKASSR-), instead of 2,3 random residues. Interestingly, sequence alignment of different NES shows that serines, lysines and arginines are tolerated mainly after the first lysine, which is exactly the position of the NLS in NC2β. The possibility that NC2β contains its NLS inside the NES is quite intriguing. There is currently no functional evidence for the existence of a NES in NC2β, although the observation that NC2β localizes also in the cytoplasm, whereas NC2α accumulates exclusively in the nucleus, could support this possibility. Interestingly, the NLS is followed by two serines, which are potential phosphorylation sites. Many important regulatory proteins, including cell cycle regulators and transcription factors, contain a phosphorylation site within or adjacent to a classical NLS (Jans 1995; Hood and Silver 1999; Jans et al. 2000). Usually dephosphorylation exposes one or more NLS and induces nuclear import (*e.g.* NFAT; Zhu et al. 1998), whereas phosphorylation leads to the exposure of an NES resulting in nuclear export mediated by the exportin protein Crm1 (*e.g.* NFAT; Zhu et al. 1998; Zhu and McKeon 1999; Macian et al. 2001. FKHRL1, a Forkhead family of transcription factors; Biggs et al. 1999; Brunet et al., 1999; Brunet et al., 2001a). It is possible that the hypothetical NC2β NES is activated after phosphorylation of the serines, whose negative charge can neutralize the positive charges of the NLS and unmask the NES. Modification of one or two serines could define two forms: one competent for nuclear import (serine unphosphorylated) and one recognized by an export factor (serine phosphorylated). Moreover, the NLS coincides with a DNA binding region and is followed by the TBP interaction domain (aa 113-133). The two serines (105-106) reside between these two domains: phosphorylation of these residues could release NC2 from the TBP-DNA complex, due to the repulsion with the negative charges of the DNA, and at

the same time unmask the NES, leading to export of the NC2β subunit.
These serines are phosphorylayion sites for CKII, PKA and, more interestingly, PKC (see Fig. 2b). In contrast to CKII and PKA, which are constitutively active kinases, PKC is activated upon phorbol ester stimulation. It could be that one or two of these serines residues are phosphorylated in certain conditions, and promote dissociation and export of NC2.
To test the hypothesis that phosphorylation at these residues can promote export of NC2β, the two serines were mutated into alanine, a neutral amino acid, or into aspartate, to mimic the negative charge acquired by phosphorylation, and the resulting mutants were tested in immunofluorescence. Mutants and wt protein had the same localization, with the protein accumulating in the nucleus in both cases, suggesting that an eventual phosphorylation at these residues did not affect protein localization. Recently, it has been shown that phosphorylation at a site adjacent to an NLS decreases the binding affinity of the NLS for the importin-α (Harreman et al. 2004). Interestingly, in this paper the authors substituted a serine at the N-terminal of an NLS with glutamate, whose negative charge is more exposed than aspartate, and they did not observe a clear cytoplasmic retention of the mutant, except when they used a yeast strain whose importin-a is defective in the import of NLS cargoes (Harreman et al. 2004)). This result would suggest that phosphorylation of serines 105-106 in NC2β could affect the functionality of the NLS or activate a potential NES, although we did not see a clear effect in our immunofluorescence experiments. Perhaps one should mutate the serines into glutamate to see an effect or use also *in vitro* assays measuring the strength of the interaction between the cargo and the importin-a or the exportin protein Crm1.

1.4. Import and dimerization

NC2α and NC2β exert their function of transcriptional repressor as a dimer. Both subunits contain a histone fold dimerisation domain similar to the histones H2A and H2B, through which they form the stable dimer. Hence, the discovery that both subunits contain an NLS was intriguing because this suggested two possible ways of import: (i) both subunits enter into the nucleus alone, where they finally dimerize; (ii) the proteins dimerize in the cytoplasm, as soon as they are translated, and only then they are imported into the nucleus (see Fig. 11a).
Interestingly, we found that when both subunits are overexpressed and thus simultaneously present in the cytopolasm, they are both retained in the cytoplasm whenever one lacks a functional NLS, even though the other had the ability to

enter the nucleus alone. This clearly indicates that, whenever NC2α and NC2β are expressed at the same time, (i) they dimerize immediately in the cytoplasm and they are imported into the nucleus as a dimer; (ii) both NLS are essential for the dimer to enter the nucleus. However, these findings do not exclude that a certain amount of the subunits can be imported separately.

1.5. Localization of NC2α in the nucleoli: site of storage in conditions where NC2α is more abundant or mechanism to regulate NC2 transcriptional repression activity?

Endogenous NC2α localizes in the nucleoplasm and, in most cells, is excluded from the nucleoli. However, when HeLa cells were transfected with NC2α constructs, the overexpressed protein strongly accumulated in the nucleoli. This phenomenon is independent from the tag fused to the N-terminal of NC2α, and is prevented when cells are co-transfected with the partner NC2β, indicating that as a dimer, NC2 never localizes in the nucleoli. These results suggest that the exogenous NC2α accumulates in the nucleoli when in high concentrations compared to the endogenous protein and to the partner NC2β. Interestingly in specific circumstances the endogenous NC2α is also observed in the nucleoli. In particular, monitoring of NC2α in different phases of the cell cycle showed that during the late G1 phase some of the cells have stained nucleoli. Several observations indicate that NC2 is not involved in repression of Pol I genes (White et al. 1994; Geisberg et al. 2001) and therefore exclude that the accumulation of NC2α in the nucleoli could be directly related to Pol I transcriptional repression. Curiously, a comprehensive characterization of proteins composing the nucleoli, did not detect NC2α (Andersen et al. 2002), likely because the proportion of cells having nucleolar NC2α is underrepresented in a population of asynchronous cells.

The nucleolus is a plurifunctional structure and its major functions are rRNA transcription and processing, and ribosome assembly (Pederson 1998). In the last years it became evident that nucleoli have also additional functions, i.e. being storage place for several proteins, whose activity is regulated by shuttling between the place where they accomplish their function and the nucleoli (Visintin et al. 1999). Likely, also NC2α accumulation in the nucleoli is related to a regulation mechanism. We observed this phenomenon during the G1 phase, when transcription is supposed to be most active, suggesting that NC2 repression activity is weakened or relieved by sequestrating part of the NC2α subunit into the nucleoli.

Consistent with the above model, mutation of threonine 23 (T23) into aspartate, a negatively charged amino acid mimicking the phosphorylation state, abolished nucleolar localization, whereas mutation into alanine, a neutral residue, had no effect. T23 is conserved in yeast and phosphorylation at this residue is crucial for transcriptional repression activity of yeast NC2 in exponentially growing cells (Creton et al. 2002). Phosphorylation at this residue can be one of the mechanisms to regulate the level of NC2α repression, controlling the shuttling of NC2α between the storage site (nucleoli) and the nucleoplasm.

Several studies raised the possibility that NC2α and NC2β also have separate functions. In yeast, Collart and colleagues demonstrated that NC2α and NC2β are not always associated in a tight complex, depending on growth conditions (Creton et al. 2002). Interestingly, the NC2 subunits show different tissue distributions (Mermelstein et al. 1996) and NC2α plays an essential function in developing of mouse embryos, independent of NC2β (Iratni et al. 2002). Very recently it has been shown that NC2α, but also the complex, associates with BTAF1, another general repressor of Pol II transcription (Klejman et al. 2004). If NC2α could have a function independently from the dimer, it could be related to its nucleolar localization.

NC2α has a bipartite NLS, with the second motif playing only a minor role in the subunit nuclear localization and no role in the import of the dimer. Notably, mutation of either the first or the second motif abolished nucleoli localization. Interestingly, NC2α accumulates in the nucleoli only when in excess compared to NC2β, namely when is not dimerized and thus also the second motif of the NC2α NLS is exposed. The consensus sequence for the nucleolar localization signal (NoLS) has not been defined yet, rather suggesting that the nucleolar targeting is mediated by the variety of molecular interactions, i.e. the specific nature of the nucleolar interaction partner (Scheer and Hock 1999). The NoLS associated with most viral proteins is usually no longer than 20 amino acids residues and possesses at least nine basic residues, including one continuous stretch of four basic amino acids or two stretches of three basic amino acids (Rowland and Yoo 2003). A NLS is usually embedded within the NoLS peptide sequence (Kubota et al. 1999). A comparison with known NoLS (Thebault et al. 2000) revealed that the NC2α N-terminus contains a stretch of basic residues similar to that found in proteins that preferentially localize in the nucleolus. Thus, our data suggest that NC2α contains a NoLS.

1.6. BiP: a good candidate NC2α-interactor localizes in the nucleoli

Biochemical studies identified several potential NC2-binding proteins. In two independent experiments, HeLa nuclear extracts and whole cell extracts were depleted with the NC2α specific antibody and all additional proteins coimmunopurified with NC2 were analyzed by MS. The protein sets obtained from the two independent experiments, performed with slightly different IP conditions, were compared. The proteins found in the first analysis were not confirmed in the second one, except for one, the heat shock 70kD protein 5 (glucose-regulated protein, 78kD, GRP78 or BiP). BiP belongs to the HSP70 family and is involved in the folding and assembly of proteins in the endoplasmic reticulum (Steel et al. 2004). However, a large proteomics analysis of the nucleolus, identified BiP ***also has a component of the nucleolus together with other unexpected proteins (Andersen et al. 2002). These observations are quite exciting because one could hypothesize that BiP is the chaperon protein that anchors NC2α in the nucleoli or assists nucleo-cytoplasmic shuffling.***

1.7. Ig-NC2α and Ig-NC2βx behave as dominant negative constructs

The fusion of a tag of ca 30kD, the Ig-tag, to both NC2 subunits blocks entry of the proteins into the nucleus by simple diffusion. Thus, when the functionality of the NLS is impaired, both proteins are retained in the cytoplasm. This is seen with the Ig-NC2α construct, were the N-terminal tag probably masks the NLS, The Ig-NC2βx deletion mutant is probably retained because of the large size and the absence of the NLS. These observations have important implications. Both NC2 subunits are essential for viability in yeast (Xie et al. 2000) and NC2α-KO mouse embryos are not viable (Iratni et al. 2002). This makes it difficult to study the effect of absence or decreased level of NC2 in metazoan systems. In yeast, the problem has been solved by substituting the endogenous NC2 with the human counterpart (Kim et al. 1997; Xie et al. 2000). The Ig-NC2α and Ig-NC2βx constructs could offer a solution to that problem. In fact, when one of the Ig-fusion proteins is overexpressed in the cell, most of the endogenous partner would be sequestered in the cytoplasm from the exogenous protein, affecting the normal NC2 balance. Thus, these fusion proteins deficient in nuclear translocation could behave like a dominant negative and could represent a powerful tool for studying effects on transcription consequent to altered NC2 levels, with respect to both subunits.

2. The mitotic phosphorylation

During mitosis, chromatin is stripped of proteins, in order to allow efficient chromosome condensation (Martinez-Balbas et al. 1995; Parsons and Spencer 1997).
Concurrently, transcription by all three RNA polymerases is inhibited (Prescott and Bender 1962; Fink and Turnock 1977).
Several findings suggest that phosphorylation plays a major role in regulating this process. Mitosis specific phosphorylation has been reported to interfere with the DNA binding activity of some transcription factors, and to exclude from mitotic chromosomes two components of the human SWI-SNF chromatin remodeling complex (Segil et al. 1991; Luscher and Eisenman 1992; Martinez et al. 1995; Sidorova et al. 1995; Muchardt et al. 1996; Segil et al. 1996).
At the same time, phosphorylation of the CTD and of the cdk7 subunit of TFIIH repress basal transcription by RNA Pol II (Cisek and Corden 1989; Zhang and Corden 1991; Leresche et al. 1996; Akoulitchev and Reinberg 1998; Long et al. 1998; Patturajan et al. 1998). Also TFIID components TBP and TAFs are phosphorylated in mitosis, and this event correlates with the inactivation of the activator-depenent transcription *in vitro* (Segil et al. 1996). Although TBP alone can support basal transcription activity *in vitro*, activator-dependent transcription requires the presence of TAFs (Burley and Roeder 1996). Thus, TAFs, but not TBP are the relevant targets for the inactivation of Pol II, (Segil et al. 1996). Similarly, phosphorylation of TBP, as a component of TFIIIB, is not responsible for Pol III transcriptional inhibition (Gottesfeld et al. 1994; White et al. 1995). Hence, there is no evidence for the functional significance of TBP phosphorylation during mitosis.

In this study, we provided evidence for the presence of mitosis specific phosphorylation of NC2α, one of the two subunits composing NC2. This is in line with an earlier report on mitotic phosphoproteins in Xenopus, which listed XNC2α as one of the proteins specifically phosphorylated in mitosis (Stukenberg et al. 1997). This observation relied on *in vitro* translation and modification by mitotic extracts that produced a mobility shift comparable to the one we observed in hNC2α.

2.1. The hyperphosphorylated NC2 does not dissociate from the condensed mitotic chromatin

Previous reports showed that the pattern of DnaseI-hypersensitive sites, which are located primarily in promoter or enhancer regions, appear to be preserved in mitotic chromosomes (Kuo et al. 1982; Kerem et al. 1984). The persistence of transcription factor footprints during mitosis varies from promoter to promoter (Hershkovitz and Riggs 1995; Martinez-Balbas et al. 1995; Wells et al. 1996; Christova and Oelgeschlager 2002). In yeast, highly condensed transcriptionally silent heterochromatin is permissive to general transcription factor binding (Sekinger and Gross 2001). Thus, many lines of evidence support the notion that chromatin structure, and therefore some promoter proteins, are retained on the chromosomes during mitosis. Indeed, several transcription factors are maintained on metaphase chromosomes, including the Serum Response Factor, the GAGA factor and AP-2 (Raff et al. 1994; Martinez-Balbas et al. 1995).

Segil et al. (1996) showed that, following nuclear envelope breakdown, 10-20% of the TFIID population remains tightly associated with the condensed mitotic chromosomes. Recently, chromatin immunoprecipitation assays confirmed that TFIID and TFIIB can remain associated with active gene promoters during mitosis, while RNA Pol II is displaced from the condensed mitotic chromosomes (Christova and Oelgeschlager 2002). Consistently, the dynamics/distribution of TBP in living human cells showed that a small fraction of TBP-TAF complexes stably associate with the condensed chromosomes during mitosis (Chen et al. 2002).

Our immunofluorescence studies showed a massive relocation of NC2α during mitosis, from the interphase nuclei to the cytoplasm. As mentioned before, TBP itself and some of the TAFs are phosphorylated in mitosis, but nevertheless they still associate with the condensed mitotic DNA. It is impossible to exclude that a small fraction of NC2α is maintained on condensed chromosomes. Indeed, a previous report showed that NC2 is maintained on some promoters during mitosis (Christova and Oelgeschlager 2002). In line with this finding, we also saw that M-NC2α maintains the ability to dimerize with the partner NC2β and bind TBP-DNA complexes. Interestingly, this activity was not impaired when nucleosome DNA was used as a template, suggesting that M-NC2-TBP-can bind also to nucleosome templates.

In conclusion, NC2, like its partner TBP, is phosphorylated in mitosis, and this modification does not preclude its ability to bind to the DNA.

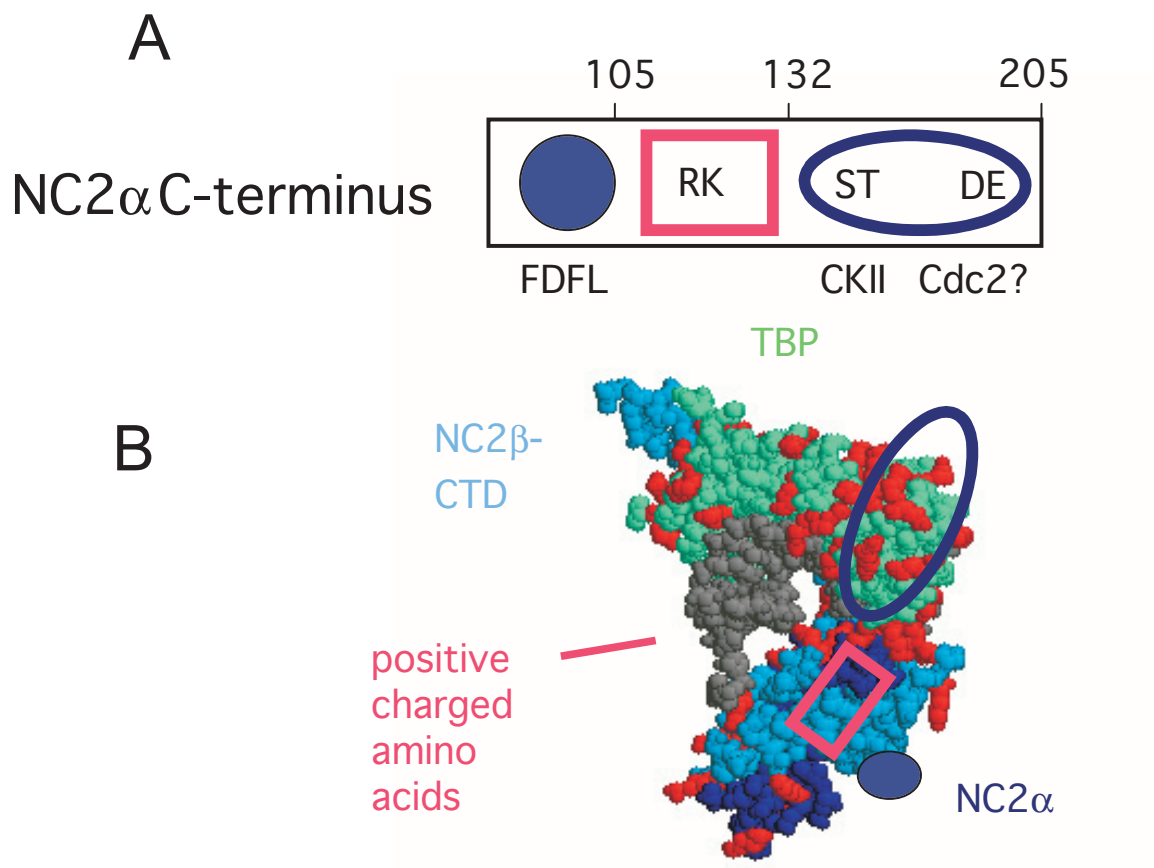


Figure 50. A model for positioning of NC2α C-terminus. (A) Schematic representation of the C-terminus of NC2α is given. Representative residues are indicated: FDFL = FDFL domain; RK = basic region; ST + DE = acidic region containing most of the potential phosphorylation sites. (B) Three-dimensional representation of the NC2-TBP-DNA complex. The strand of DNA is in gray; TBP in green (red corresponds to the basic residues); NC2β in light blue; NC2α dark blue. Oval-blue shapes correspond to the acidic terminal domain of NC2α. Two possible positioning of the tail are shown, contacting either the basic surface of TBP or the DNA.

2.2. Hypothetical functions of mitotic phosphorylation

Previous studies suggested that phosphorylation of NC2 by CKII blocks its non-specific interaction with DNA. Most of the potential phosphorylation sites of NC2α are located in the C-terminus. The C-terminus of NC2α was missing in the NC2-TBP-DNA crystal structure (Kamada et al., 2001). This region contains two acidic regions spaced by a proline rich domain (see Fig. 2a and 2b). Interestingly, most of the potential CKII phosphorylation sites reside in the first acidic domain. By looking at the crystal structure one may hypothesize that the C-terminus bends towards TBP and the acidic region enters in contact with an identified positively charged surface of TBP (Fig. 28). The electrostatic interaction between opposite charges would strengthen the trimeric complex, with TBP-NC2 resembling a ring-

like structure around the DNA. Support for this model comes from the recent observations that the first acidic region of the NC2α C-terminal contains an additional potential TBP interaction domain (Gilfillan, unpublished data; Klejman et al. 2004). Deletion-mutants showed that the first acidic stretch is involved in stabilizing the complex, especially in the absence of the NC2β C-terminus. Interestingly, this region contains many of the potential phosphorylation sites. The mitotic specific NC2 hyperphosphorylation can therefore participate in making the interaction more stable.

Alternatively, the C-terminus phosphorylation could impair interaction with other proteins, like activators or in general factors that relieve NC2 repression activity, so that during mitosis part of NC2 remains stably associated with TBP-DNA. Very recently, it has been published that NC2α tail interacts with BTAF1, enhancing its binding to TBP (Klejman et al. 2004), although NC2 phosphorylation by CKII was not required for this function.

3. Effect of normal NC2 phosphorylation

Previous studies showed that phosphorylated NC2α does not bind to the DNA. (Goppelt et al. 1996). Our results on binding ability to long and nucleosome template DNA imply that recombinant unmodified NC2 and native phosphorylated NC2 behave differently in complex formation: the native NC2-TBP-DNA complex has a lower DNA binding capacity. This suggests that the polymerization of TBP-NC2 is inhibited by electrostatic repulsion.

4. Both NC2α and NC2β have several isoforms

Separation of NC2 native proteins in 2D gels clearly showed for the first time that both NC2α and NC2β have many unexpected isoforms. Five distinct spots corresponding to NC2α were identified, among which only one is the mitotic-specific form. The 2D pattern is compatible with differentially phosphorylated modifications.

Seven different spots were identified as NC2β, four of which were particularly

abundant (spots 1 to 4 in Fig. 26).
The NC2β band normally seen in WB is split in three spots that likely differ for the phosphorylation status (spot n.2, 3, 4). Moreover a new previously unidentified band of NC2β with lower molecular weight was also found (spot n.1). Size differences point to a deletion form of NC2β. Interestingly, the silver stain suggested that this form was as abundant as the others, although normally it is not recognized by the antibody. This protein became visible also when NC2 was marked with radioactivity. A possible explanation could be that our NC2β antibody does recognize this shorter form, as proven by the clear signal given in the 2D WB, but of course in a less efficient way than the whole protein.

4.1. The high mobility NC2β form is likely a deleted protein

The high mobility NC2β form could be a C-terminal deletion protein, rather than the unmodified version of the usual NC2β form. First, mass spectrometry analysis of the 2D NC2β spots identified most of the peptides within the amino acids 1-135 and no difference at all among the 4 major spots, suggesting that the mobility difference could be explained in the C-terminal unidentified part of the protein. Second, this form coimmunoprecipitates with NC2α. Since the first 80 amino acids of the histone fold domain are essential for protein-protein interaction, the short NC2β must contain them.
This protein was observed in all the three extracts, arguing against an artefact, but one can not exclude that it comes from protein degradation.

V. MATERIALS AND METHODS

1. Materials

1.1. Chemicals and biochemicals

Acetic Acid	Roth
Acrylamide/Bisacrylamide 30% (Rotiphorese Gel 30)	Roth
Acrylamide solution 30% (Rotiphorese Gel A)	Roth
Agarose	Gibco
Ammonium persulfate (APS)	Merck, Roth
Ammonium sulphate	Merck
Ampicillin (Ap)	Roth
Aprotinin	Sigma
Bacto Agar	Difco
Bacto Trypton	Difco
Bacto Yeast Extract	Difco
Benzamidin	Sigma
Bisacrylamide solution 2%	Roth
Boric acid	Roth
5-Bromo-4-Chloro-3-indolyl-phosphate (BCIP)	Peqlab
Bromophenol Blue	Sigma
BSA (10 mg/ml) (bovine serum albumin)	New England Biolabs
Calciumchloride	Merck
Calciumhydronenphosphate	Merck
Calciumhydroxide	Merck
CHAPS	Sigma
Chloroform	Merck
Coomassie brilliant blue R-250	Sigma
Dimethylsulfoxide	Sigma
Dithiothreitol (DTT)	Roth
DMEM medium	Gibco
dNTPs	Roche
Ethanol (EtOH)	Nardini
Ethanolamine	Sigma
Ethidium bromide	Sigma
Ethylendiamintetraacetate disodium salt (EDTA)	Merck
Fetal calf serum (FCS)	Gibco
Fish gelatine	Sigma

Glucose	Merck
Glycerol	Roth
Glycine	Roth
Hepes	Biomol
Histogel mounting medium	Linaris
Isoamyl alcohol	Merck
Isopropanol	Merck
Leupeptin	Roche
Magnesiumchloride	Merck
β-Mercaptoethanol	Sigma
Methanol	Merck, Roth
Milk powder	Heirler Cenovis GmbH
Mimosine	Sigma
Nitro-blue-tetrazolium (NBT)	Peqlab
Nocodazole	Sigma
Okdaic acid	Sigma
Paraformaldehyde	Merck
Penicillin-Streptomycin	Invitrogen
Phenol/chloroform	Roth
Phenylmethylsulfonfluoride (PMSF)	Biomol, Roth
Phorbolmyristylester (PMA)	Roche, Sigma
Ponceau S	Sigma
Propidium iodide	Sigma
Protein G-Sepharose	Amersham
RPMI 1640-Medium	Gibco
Sodium azide	Sigma
Sodium borate	Roth
Sodium carbonate	Merck
Sodium chloride	Monopolio di Stato (Saline di Favignana)
Sodiumdodecylsulphate (SDS)	Merck, Roth
Sodium fluoride	Sigma
Sodium hydroxid	Merck
Sodium-hort-vanadate	Sigma
Sucrose	Sigma
Tetramethylethylendiamin (TEMED)	Sigma
Thymidine	Sigma
Trishydroxidmethyl-aminomethan (Tris)	Sigma
Triton X-100	Sigma
Trypsin-EDTA solution	Gibco
Urea	Roth
Xylene cyanole	Fluka

1.2. Additional material

Disposable plastic material	Greiner, Nunc, TPP, Falcon
ECL Western Blot Kit	NEN
Film X-OMAT, BioMax	Kodak
Gel Drying Kit	Promega
GFX Gel Band Purification Kit	Amersham
IEF-Gel strips (IPG)	Amersham
Nitrocellulose membrane (0.45 μm mesh)	BioRad
Nucleobond AX Plasmid DNA Kit	Macherey & Nagel
PVDF-Membrane Hydrobond-P	Amersham
Siliconized Plastic tubes	Sorenson
Silver Staining Kit	PlusOne, Amersham
Sterilfilter (0.22/0.45 μm)	Roth
Concentrator (MWCO 10 kD)	Vivaspin 2, Vivascience
Whatman 3MM Paper	Whatman

1.3. Instruments

2D-Gel electrophoresis system	IPGphor / Ettan Dalt, Amersham
Acrylamid gel electrophoresis	Amersham / Hoefer SE280, SE600, SE660
Agarose gel electrophoresis	BioRad
Autoradiography cassette	Amersham, Kodak
Developing machine	Hyperprocessor, Amersham
Electroblot-Apparatus (semi-dry)	BioRad
Gel drier	GD2000, Hoefer
Homogenizer	Douncer, Wheaton
Incubator	WJ311, Forma Scientific
	Unequip, Unitherm
	B6200, Heraeus
Instant Imager	Packard
Confocal light microscope	TCS SP2 and TCS, Leica
Light microscope	Axiovert 25, Zeiss
MALDI-TOF/TOF-MS	Proteomics Analyzer 4700, Applied Biosystems
PCR-Thermocycler	GeneAmp 2400, Applied
pH-Meter	Calimatic 760, Knick
Photometer	GeneQuant Pro, Amersham
Rotoren	JA10, JA25-50, SW41, SW28, Beckman
Ultra-centrifuge	L7, L8-M von Beckman
UV-Illuminator	Bachofer (254 nm, 366 nm)
Centrifuges	Avanti, Beckman
	Multifuge 3 L-R, Heraeus
	Zentrifuge 5417, 5415R, Eppendorf

1.4. Enzymes

Enzyme	Company
Klenow fragment	Fermentas
Pfu DNA polymerase	Promega
Restriction enzymes	New England Biolabs or Fermentas
RNase A	Roche
T4 DNA ligase	New England Biolabs or Fermentas
T4 polynucleotide kinase	New England Biolabs
Taq polymerase	Fermentas

1.5. General buffers

2x Acetylation buffer:
100 mM Tris-HCl (pH 8.0 RT), 10 mM EDTA (pH 8.0), 300 mM NaCl, 1% NP40 (v/v). Add freshly 1 mM DTT, protease inhibitors (0.2 mM PMSF, 1 mM Benzamidine) and deacetylation inhibitor (Butirrate 0.1 mM).

Annealing buffer for oligonucleotides used in EMSA:
10 mM Tris-HCl (pH 7.3 RT), 200 mM NaCl, 1 mM $MgCl_2$.

AP-buffer (Alcaline Phosphatase reaction):
100 mM Tris-HCl (pH 8.8 RT), 100 mM NaCl, 5 mM $MgCl_2$..

Bacterial lysis buffer:
500 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.3, 5 mM β-Mercaptoethanol, 0.1% IGEPAL CA-630 (NP-40).
Add fresh protease inhibitors to final concentration: 2 μg/ml Leupeptin, 1 μg/ml Pepstatin A, 20 μg/ml Benzamidine and 1 mM Phenylmethylsulfonyl fluoride.

BCx-buffer:
20 mM Tris-HCl (pH 7.3 RT), 0.2 mM EDTA, 20% (v/v) Glycerine, x mM Kcl.

5x Cell colture lysis buffer:
125 mM Tris-HCl (pH 7.8 RT), 10 mM EDTA, 50% (v/v) glycerine, 5% (v/v) Triton-X, 10 mM DTT. Filter sterilize through a 0.45-μm nitrocellulose filter (Millipore). Store in aliquots at -20 °C. Dilute with PBS to a 2x working solution.
Add fresh protease inhibitors (200 mM PMSF, 100 mM Benzamidine, 2 μg/ml

Leupeptin, 2 μg/ml Aprotinin, 0.2 μg/ml Pepstatin A) and phosphatase inhibitors (1 mM NaF, 100 mM Vanadate).

HB-buffer (Hypotonic Buffer):
For 1 l buffer use 10 ml 1 M Tris-HCl (pH 6.8 RT), 1.5 ml 1 M $MgCl_2$, 3.34 ml 3 M KCl.

2x HBS (Hepes-buffered saline):
280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4. Adjust to pH 7.05 with 0.5 N NaOH at RT. Filter sterilize through a 0.45-μm nitrocellulose filter (Millipore). Store in aliquots at -20 °C.

High Salt Buffer (1.6 M):
For 1 l buffer use 20 ml 1 M Tris-HCl (pH 6.8 RT), 250 ml Glycerin, 533 ml 3M KCl, 1.5 ml 1M $MgCl_2$, 0.4 ml 0.5 M EDTA.

Low Salt Buffer (0.02 M):
For 1 l buffer use 20 ml 1 M Tris-HCl (pH 6.8 RT), 250 ml Glycerin, 6.67 ml 3M KCl, 1.5 ml 1M $MgCl_2$, 0.4 ml 0.5 M EDTA.

20 x PBS:
160 g NaCl, 4 g KCl, 36 g $Na_2HPO_4 \cdot 2\ H_2O$, 4.8 g KH_2PO_4, pH 7.4, add dH_2O to 1 l.

Transfer buffer:
3 g Tris base, 14.4 g glycine, in 800 ml dH_2O. Add 200 ml MeOH before use.

10 x TBE:
1 M Tris, 1 M boric acid, 20 mM EDTA (pH 8.0 RT).

10 x TBS:
24.2 g Tris base, 80 g NaCl, 2 g KCl. Adjust pH to 7.6 with HCl concentrated, add dH_2O to 1 l.

1 x TBST:
1 x TBS, 0.05% (v/v) Tween 20.

10 x TGE:
30 g Tris base, 186 g glycine, 20 ml 0.5 M EDTA in 1 l dH_2O.

10 x TGS:
250 mM Tris, 1.92 M Glycin, 1% (w/v) SDS.

TE:
10 mM Tris-HCl (pH 8.0 RT), 1 mM EDTA (pH 8.0).

Sample Buffer:
1 g glucose in 1 l PBS.

Staining / Distaining buffer:
To 500 ml H_2O add 400 ml MeOH and 100 ml glacial acetic acid. For staining, add 2.5 g Coomassie Brillant Blue R250 for 1000 ml of distain solution.

1.6. Antibodies (ab)

1.6.1. Primary antibodies

β-Actin sc-1616 (I-16)	Goat polyclonal	SantaCruz Biotechnology
NC2α 4G7	Rat monoclonal	E. Kremmer, Munich
NC2α 6G8	Rat monoclonal	E. Kremmer, Munich
NC2β	Rabbit polyclonal	M. Meisterernst
TBP sc-273	Rabbit polyclonal	SantaCruz Biotechnology
Flag M2 F3165	Mouse monoclonal	Sigma

1.6.2. Secondary antibodies

Sheep anti-Maus IgG H+L (515-095-062)	FITC-conjugated	Jackson
Sheep anti-Mouse IgG (H+L) (515-095-063)	FITC-conjugated	Jackson
Goat anti-Human IgG (H+L) (109-095-107)	FITC- conjugated	Jackson
Mouse anti-Rabbit IgG (H+L) (211-095-109)	FITC- conjugated	Jackson
Mouse anti-Rat IgG (H+L) (212-066-102)	Biotin-SP-conjugated	Jackson
	Alexa488-SP-conjugated	Molecular Probes
	Cy3- SP-conjugated	Jackson
Goat anti-Rat IgG + IgM (112-166-068)	Cy3-conjugated	Jackson
Goat anti-Rat IgG H+L (S3831)	AP-conjugated	Promega
Goat anti-Rat IgG H+L (112-035-068)	HRP-conjugated	Jackson
Donkey anti-Goat IgG (sc-2022)	AP-conjugated	SantaCruz
Goat anti-Mouse IgG H+L (S3721)	AP-conjugated	Promega
Goat anti-Rabbit IgG Fc (S3731)	AP-conjugated	Promega
Goat anti- Rabbit IgG H+L (W4011)	HRP-conjugated	Promega
Goat anti- Mouse IgG H+L (W4021)	HRP-conjugated	Promega
Donkey anti- Goat IgG H+L (705-035-147)	HRP-conjugated	Dianova

1.7. List of plasmids

Plasmid	Description	Backbone	Origin
pcDNA3		pcDNA3	Invitrogen
pGS49	His-NC2α	Rc/CMV	M. Meisterernst
pGS46	His-NC2β	Rc/CMV	M. Meisterernst
pGS67	Ig-NC2α	CDM7	M. Meisterernst
pGS68	Ig-NC2β	Rc/CMV	M. Meisterernst
	Ig-NC2βy	Rc/CMV	M. Meisterernst
PGS163	Ig-NC2βx	Rc/CMV	M. Meisterernst
pSO5	His-NC2α-His-NC2β	pET11d.1	Gilfillan et al., 2003
pB2-MLP	AdML promoter		M. Timmers

2. Methods

2.1. Cloning and related techniques

2.1.1. Cloning of NC2 constructs

pEP2 (pcDNA3-Flag)

pEP2 was constructed by two fragment ligation of an HindIII, KpnI fragment derived from annealing of two oligos, and a HindIII, KpnI cut vector derived from pcDNA3. The sequences of the oligos are:

HindIII *Kozak* Flag-tag NdeI KpnI

flag.for: 5'-**AGCTT**A*CCATGG*ACTACAAAGACGATGACGACAAGGGTCATATG**GGTAC**-3'

fleg.rev: 5'-CCATATGACCCTTGTCGTCATCGTCTTTGTAGTCCATGGTA-3'

pEP3 (Flag-hNC2α in Rc/CMV)

pEP3 was constructed by two fragment ligation of an NdeI fragment derived from pEP2 and a NdeI fragment derived from the vector pGS49

pEP4 (Flag-hNC2β in Rc/CMV)

pEP4 was pEP3 was constructed by two fragment ligation of an NdeI fragment derived from pEP2 and a NdeI fragment derived from the vector pGS46.

2.1.2. Point mutants generation

The point mutants in the NLS sequences were introduced following the instructions

of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).
pGS49 was used as template for construction of the following plasmids: pEP31 pEP33, pEP35 and pEP36.
pEP4 was used as template for construction of the following plasmids: pEP13, pEP11 and pEP30.
PEP 31 was used as template for construction of the pEP32 plasmid.
PEP 33 was used as template for construction of the pEP34 plasmid.

The sequences of the oligos used for the mutagenesis are listed below.

Plasmid: pEP31 (His-hNC2α(K4A/K5A) in Rc/CMV)
Primers: aK4/5A.for
5'-GGCAGCCATATGCCCTCCGCGGCGAAAAAGTACAATGCCCGG-3'
aK4/5A.rev
5'-CCGGGCATTGTACTTTTTCGCCGCGGAGGGCATATGGCTGCC-3'

Plasmid: pEP32 (His-hNC2α(K4A/K5A/K6A/K7A) in Rc/CMV)
Primers: a.K6/7Apost.for
5'-CATATGCCCTCCGCGGCGGCAGCGTACAATGCCCGGTTCCC-3'
a.K6/7Apost.rev
5'-GGGAACCGGGCATTGTACGCTGCCGCCGCGGAGGGCATATG-3'

Plasmid: pEP33 (His-hNC2α(K18A/K19A) in Rc/CMV)
Primers: aK18/19.for
5'-GTTCCCGCCGGCGCGGATCGCGGCGATCATGCAGCAGGACG-3'
aK18/19.rev
5'-CGTCCGTCTGCATGATCGCCGCGATCCGCGCCGGCGGGAAC-3'

Plasmid: pEP34 (His-hNC2α(R16A/K18A/K19A) in Rc/CMV)
Primers: a.R16Apost.for
5'-GGTTCCCGCCGGCGGCGATCGCGGCGATCATG-3'
a.R16Apost.rev
5'-CATGATCGCCGCGATCGCCGCCGGCGGGAACC-3'

Plasmid: pEP36 (His-hNC2α(T23A) in Rc/CMV)
Primers: a.T23A.for
5'-GAAGATCATGCAGGCGGACGAAGAGATTGG-3'
a.T23A.rev
5'-CCAATCTCTTCGTCCGCCTGCATGATCTTC-3'

Plasmid: pEP35 (His-hNC2α(T23D) in Rc/CMV)
Primers: a.T23D.for
5'-CAAGAAGATCATGCAGGACGACGAAGAGATTGGG-3'
a.T23D.rev
5'-CCCAATCTCTTCGTCGTCCTGCATGATCTTCTTG-3'

Plasmid: pEP13 (Flag-hNC2β(R101A/R102A) in Rc/CMV)
Primers: b.RR101-102A.for
5'-ACAGTAGCATTAAAAGCAGCAAAGGCCAGTTCTCGTTTGG-3'
b.RR101-102A.rev
5'-CCAAACGAGAACTGGCCTTTGCTGCTTTTAATGCTACTGT-3'

Plasmid: pEP11 (Flag-hNC2β(S105A/S106A) in Rc/CMV)
Primers: b.S105/106A.for
5'-AGAAGAAAGGCCGCTGCTCGTTTGGAAAACCTTGGC-3'
b.S105/106A.rev
3'-GCCAAGGTTTTCCAAACGAGCAGCGGCCTTTCTTCT-3'

Plasmid: pEP30 (Flag-hNC2β(S105D/S106D) in Rc/CMV)
Primers: b.S105/106D.for
5'-AGAAGAAAGGCCGATGATCGTTTGGAAAACCTTGGC-3'
b.S105/106D.rev
5'-GCCAAGGTTTTCCAAACGATCATCGGCCTTTCTTCT-3'

All of the above PCRs were performed with PfuTurbo DNA polymerase (Promega, Madison, WI), using deoxyribonucleosidetri-phosphate mix (final concentration 250 mM) and reaction buffers, supplied by the manufacturers.

PCR conditions:

Reagent	Volume
10x enzyme buffer	5 μl
125ng of oligonucleotide primer #1	
125ng of oligonucleotide primer #2	
dNTPs (each dNTP is 25 mM)	1 μl
DNA	10 ng
ddH_2O to a final volume of 50 μl	
PfuTurbo DNA polymerase (3u/μl)	1 μl

PCR cycles:

Step	T	time
1	95 °C	Pause
2	95 °C	30s
3	55 °C	60s
4	68 °C	13m 30s
5	68 °C	10m
6	4 °C	∞

The steps 2-4 were repeated 16x.

Each amplification reaction was digested with 1 μl of the DpnI restriction enzyme (10U/μl, New England Biolab) for 2-3 h at 37 °C, 5 μl of the digest were used for transformation. All mutations were checked by sequencing.

2.1.3. Restriction digests

Restriction digests were typically done for 2 h at 37 °C with 2 units restriction endonuclease per μg DNA in the appropriate buffer as recommended by the manufacturer (New England Biolabs, Beverly, MA, or Fermentas).

2.1.4. Ligation of DNA

Ligation was done with T4 DNA ligase in buffer supplied by the manufacturer (New England Biolabs, Beverly, MA) in a final volume of 20 μl over night at 16 °C.

2.1.5. Agarose gel electrophoresis

Agarose (electrophoresis grade, GibcoBRL, Grand Island, NY) was dissolved in 1xTBE buffer to the desired concentration (0.8 to 2% depending on the size of DNA fragments) by boiling in a microwave oven, and after cooling down, ethidium bromide was added to a final concentration of 0.5 *μ*g/ml and the agarose solution poured in a gel chamber. The DNA sample was mixed 1:6 with 6xDNA sample buffer (30% glycerol, 0.25% Bromphenol Blue, 0.25% Xylene Cyanole in TBE buffer). The gels were run in 1xTBE buffer and visualized on a UV transilluminator.

2.1.6. Preparation of competent E. coli

50 ml LB medium were inoculated with 0.5 ml overnight culture of *E. coli* DH5α or MC1061 and grown to A_{600} of 0.5. The bacteria were then centrifuged in a 50 ml

conical tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at 3000 rpm (5 min at 4 °C) resuspended in 25 ml cold sterile 0.1 M $CaCl_2$ and stored on ice for 30 min. After a second spin as above, the bacteria were resuspended in 1 ml cold 0.1 M $CaCl_2$ and frozen in 15% sterile glycerol at -80 °C, in aliquots.

2.1.7. Transformation and growth of transformed bacteria

50 μl competent bacterial cells were mixed with 0.1-500 ng of plasmid DNA or 10 μl ligation and incubated on ice for 30 min. The cells were then heat shocked by placing in a 42 °C thermoblock for 45 s and then let on ice fore 2 min. 1 ml LB medium was then added to the transformation mix and the tubes were placed in a 37 °C shaker for 1h. Afterwards, 200 μl of bacterial cells were spread onto LB agar plates containing either 100 μg/ml ampicillin or 12.5 μg/ml tetracycline and 6.5 μg/ml ampicillin (for the CDM7 vectors) and grown overnight at 37 °C. Single colonies were used to inoculate either 4 ml LB for the 200 ml LB cultures, grown overnight at 37 °C, shaking at 200 rpm, and used the day after for either the mini or the maxi preps of plasmid DNA.

2.1.8.“Mini” and “maxi” plasmid preparation

Maxi preps were done using kits Nucleobond AX (Macherey & Nagel) according to the manufacturer’s instructions. Mini preps were done according to Clewell et al. (1970).

2.2. Tissues culture and related techniques

2.2.1. Cell lines

HeLa	Human epithelial cell line originating from a cervical carcinoma. Adherent cells.
HeLA S3	Derived from HeLa. Cells have been adapted to grow in suspension culture
Jurkat	Human T lymphoblastoid cell line
293T	Adenovirus 5-transformed human embryonic kidney cell line.
F99	Human skin primary fibroblast

2.2.2. Culture conditions

The cell lines used in this study were grown in the following media:

HeLa, 293T, F99 - Dulbecco's modified Eagle medium (DMEM plus 4500 mg/ml glucose, L-Glutamine, without Pyruvate. Gibco Invitrogen, Cat. No.11971-025) supplemented with 10% heat-inactivated (56 °C for 30 min in a water bath) foetal bovine (calf) serum (FBS=FCS, Gibco Invitrogen, Cat. No.10270-106), 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (Gibco Invitrogen, Cat. No.15140-122).

Jurkat and HeLA S3 - RPMI 1640 medium (plus L-Glutamine, Gibco Invitrogen, Cat. No.21875-034), supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin G, 100 *μ*g/ml streptomycin sulfate.

The adherent cell lines, used in this study were usually grown in 10-cm or 15-cm tissue culture dishes in a tissue culture incubator with 5% CO_2 atmosphere at 37 °C. Subculturing of the adherent cells was done by trypsinization with 0.25% trypsin, 0.2% EDTA (Gibco Invitrogen, Cat. No.25050-014) every second day. Cells were usually kept in culture for no more than 20 passages.

Suspension cell lines were grown in 25, 75 or 175 cm^2 tissue culture flasks. Subculturing of suspension cells was done by diluting with fresh medium. Cells were kept at concentration between 0.2-1x10^6 cells/ml.

Freezing of cells was done in 90% FCS and 10% (v/v) dimethyl-sulfoxide (DMSO) in a freezing box at -80°C. The frozen aliquots were stored in a liquid nitrogen tank or at -80°C.

Thawing of the cells was done by submerging the frozen vial in a 37 °C water bath. The cells were then washed with medium by centrifugation at 1200 rpm for 3 min and resuspended in pre-warmed medium before transferring them to culture plates or flasks.

2.2.3. Cell syncronization

Unless specified, adherent HeLa cells were used in the following synchronization protocols. Cells were maintained exponentially growing, splitting them regularly every second day. To increase the efficiency of synchronization, cells were always plated the day before to obtain dishes ca. 60% confluent when exposed to the specific inhibitors.

2.2.3.1. Mitotic block

At mitosis (M) most vertebrate cells round-up. In many cell lines, especially those of epithelial origin, which grow in monolayer, attachment to the substrate also becomes much loser. This causes some mitotic cells to detach spontaneously. The mitotic arrest can be induced chemically using nocodazole, an inhibitor of microtubule polymerization. An almost pure population of M-blocked cells can be obtained combining the nocodazole block to mechanical shaking. Cells were treated with 600 ng/ml nocodazole (Sigma) for 16 h before harvesting.

2.2.3.2. Mitotic block and release into G1

Cells were exposed to 50 ng/ml nocodazole for 17 h, the mitotic cells were collected by gentle pipetting, and washed in DMEM at 37 °C, resuspended in DMEM/10% FCS, replated and cultured for an additional 6 h. The mitotic cells obtained from four 15 cm plates were plated onto one 10 cm plate.

2.2.3.3. Late G1 block

L-mimosine is a plant aminoacid that provokes synchronization of cells just before the start of the S phase. HeLa cells were arrested in late G1 exposing them to 400 μM mimosine (Sigma) for 20 h before harvesting.

2.2.3.4. S and G2 block

Any inhibitor of DNA synthesis arrests replicating cells. The most used are inhibitors of the synthesis of deoxyribonucleotide triphosphates, as thymidine at high concentrations. A double-arrest accumulates more than 90% of the population. HeLa cells were exposed to 2 mM thymidine for 17 h, rinsed twice, released in growth medium for 9 h and then blocked again with 2 mM thymidine for 15 h. At this point cells are synchronized at late G1. To obtain a G2 population, cells were washed and let grow in normal medium for 8 h.

2.2.4. Metabolic labeling of mitotic and asynchronous cells

At 13 h after the addition of nocodazole, the mitotic detached cells of four 15 cm plates were collected, washed twice and cultured in a well of a 6-wells plate with 7 ml of H_3PO_4-free medium (Dulbecco's Modified Eagle Medium, with high glucose and L-Glutamine, without Sodium Phosphate and Sodium Pyruvate Gibco Invitrogen, Cat. No. 11971-025), supplemented with 20 mM ***Hepes*** buffer, penicillin-streptomycin, 10% Fetal Bovine Serum (FBS) Dialyzed (H_3PO_4-free. Gibco, Cat. No. 26400-036), and 600 ng/ml nocodazole. After 2 h cells were centrifugated and

resuspended in 2 ml of the same medium supplemented with [^{32}P]H_3PO_4 (0.5 mCi / 1 ml of medium) (NEN Life Science Products, Perkin Elmer, Cat. No. NEX053). Cells were labeled for 5 h before harvesting.

The sample of exponentially growing cells was exposed to H_3PO_4-free medium containing 600 ng/ml nocodazole and 10% FBS (H_3PO_4-free) for 2 h, and then 5 h more with [^{32}P]H_3PO_4. One 12 cm plate was used for the exponentially cells and the plate was covered with 2.5 ml medium (0.5 mCi / 1 ml of medium).

In order to preserve protein phosphorylations, the cell lysis buffer (see below) contained in addition 1μM okadaic acid.

2.2.5. Calcium phosphate transfection

Transfection was performed according to the protocol described in Chen and Okayama (1988).

The day before transfection, 293T or HeLa cells were seeded in 6-well plates at 30% confluency. 20 μg of total DNA (the concentration in each transfection experiment was kept constant by adding vector plasmid DNA) were added to 500 μl of 250 mM $CaCl_2$, and then the tubes were vortexed. 500 μl of 2xHBS were added dropwise while gently vortexing, and the solution was incubated 20 min at RT. The calcium phosphate-DNA solution (160 μl / well) was added dropwise onto the cell culture plate while swirling. The plates were incubated overnight in a 5% CO_2-humidified incubator at 37 °C to allow for a calcium phosphate-DNA complex to gradually form. The cells were then washed three times in PBS, and incubated in complete medium in a 5% CO_2 humidified incubator at 37 °C.

2.2.6. Polyfect transfection

Transfection of HeLa cells with PolyFect transfection reagent (Quiagen, Hilden) was conducted following the manufacturer. For each well of a 6-well plate, 1.5 μg of DNA dissolved in TE buffer was diluted in 100 μl OPTIMEM 1 (with Glutamax, Gibco Invitrogen, Cat. No. 51985-026) before adding 12 μl of PolyFect transfection reagent was added. Note that this reagent is toxic for the cells, and the day after transfection dead cells were observed in the supernatant, also in the control (cell transfected with dH_2O). Therefore cells were always fixed for the immunofluorescence 16 to 24 h after transfection.

2.3. FACS (Flow cytometry analysis)

For flow cytometry analysis, adherent HeLa or Jurkat cells were washed twice with ice-cold PBS (HeLa cells after resuspension with Trypsin/EDTA). Then, cells were counted and adjusted to a concentration of ca. $1x10^6$ cells/ml. Between 200-1000 μl of cells centrifuged (1200 rpm, 5 min), supernatant was removed and cells were resuspended by vortex in the remaining fluid. Cells were fixed adding 1 ml of ice-cold of 70% EtOH drop by drop while vortexing. Then, cells were allowed to fix overnight at 4 °C. For the flow cytometry analysis, cells were resuspended again by vortexing, centrifuged 5 min at high speed (3000 rpm) and the staining solution was added to the cells resuspended in the residual (after removing) ethanol. The volume of the staining solution (50 μg/ml propidium iodide, 100 U/ml RnaseA, 2 mM EDTA in PBS) varied according to the quantity of cells, in order to obtain a constant concentration ($1x10^6$ cells/ml). Cells were incubated for at least 30 min at RT and readily analyzed in a FACSCalibur flow cytometer (Becton Dickinson).

2.4. Immunofluorescence

Unless stated, sample preparation was done at room temperature. Antibodies and dilutions are described below. HeLa cells plated on glass coverslips (11 mm) and transfected as described above were washed once with PBS and fixed with 3.7% PFA/PBS for 10 min (see below for solution preparation). Then, they were washed twice with PBS and incubated 15 min in 1 mg/ml $NaBH_4$/PBS. After washing again twice with PBS, permeabilisation was performed with 1% Triton X-100/PBS for 15 min and then washed twice with PBS. Blocking was done with 0.2% fish gelatin (Sigma) for 20 min. A humid chamber was prepared containing one layer of thick paper moisten with water and a stripe of parafilm. Drops (50-100 μl) of primary ab dilution were pipetted over the parafilm. Each coverslip was then transferred over a single drop, with cells facing the ab, and incubated 45-60 min at 37 °C. Afterwards, coverslips were transferred again to a 6-well plate and washed three times with PBS, for at least 30 min. Incubation with the secondary ab conjugated to the fluorescent dye was carried out as above, but for 30 min and in the dark, due to the fotosensibility of the fluorescent dye. If the secondary ab was biotin conjugated, a third incubation was performed (after 3x PBS washes for 30min) identical to the second one, with streptavidine dye-conjugated, followed by three PBS washing. Last, DNA stain was performed. To get red staining cells were preincubated with 10

μg/ml RNase/PBS for 20 min at 37 °C and then with 100 μg/ml propidium-iodide for 10 min. To obtain a blue stain, cells were incubated with 1 μg/ml DAPI/PBS for 10 min. After a final wash (3x with PBS followed by dH_20) the coverslips were mounted on a precleaned microscope slide in histogel mounting medium (Linaris, Wertheim-Bettingen, Germany). In alternative, coverslips were mounted with 10% glycerol/ PBS and then sealed using nail polish. When possible, cells were observed at the microscope within one day. Slides were stored at 4 °C for two to three weeks, after which the quality diminished.

Target	**NC2α**	**NC2α**	**NC2α**	**Ig**	**Flag**	**NO38**
Proteins	NC2α	NC2α	Ig-NC2α, His-NC2α (wt and mutants)	Ig-NC2β, Ig-NC2βy, Ig-NC2βx	Flag-NC2β (wt and mutants)	NO38
Primary ab	4G7	4G7	4G7	--	M2	NO38
Dilution	1:10	1:10	1:10		1:400	1:200
Secondary ab	Biotin anti-rat	Biotin anti-rat	Cy3 anti-rat	FITC anti-h IgG	FITC anti-mouse	Texas red anti Guinea Pig
Dilution	1:300	1:300	1:400	1:100	1:100	1:200
Streptavidine (optional)	Cy3-streptavidine	Alexa 488	--	--	--	--
Dilution	1:400	1:400				
Color	Red	Green	Red	Green	Green	Red

Fixation solution:

37% paraformaldehyde (10x PFA):

1.85 g PFA, 3.5 ml dH_2O, 10 μl 10 M KOH.

Mix together in a 50 ml Falcon tube. Boil water in a glass beaker in the microwave and put tube in it with cap loose, swirling frequently to mix, for no longer than 5 min, until the PFA goes into solution. Store at -20 °C.

2.4.1. Confocal microscopy

In conventional fluorescence microscopy, fluorescent light emitted by a sample comes from molecules above and below the plane of focus, thus the observer sees

a blurred images caused by the superposition of fluorescent images from molecules and many depths in the cell. The blurring effect makes it difficult to determine the actual tree dimensional molecular arrangement. Confocal scanning microscopy produces much sharper images by reducing the image-degrading effects of out-of-focus light. Exciting light from a focused laser beam illuminates only a single small part of a sample for an instant and then rapidly moves to different spots in the same focal plane. The emitted fluorescent light passes through a pinhole that rejects out of focus light, thereby producing a sharp image. Because light in focus with the image is collected by the pinhole, the scanned area is an optical section through the specimen. The intensity of light from these in-focus areas is recorded by a photomultiplier tube, and the image is stored in a computer.

Microscopy was done with two confocal microscopes:

1) confocal microscope Leica TCS (Leica, Bensheim) equipped with an Ar-Kr laser (488 nm and 568 nm) exciting both FITC (green) and Cy3/PI (red). When double transfection experiments were performed, cells were stained with only one dye at the time, to avoid cross-talk. In fact, the emission of the FITC can excite red, resulting in a false positive Cy3 signal.

2) confocal laser-scanning-microscope Leica TCS SP2 (Leica, Bensheim) equipped with four lasers: (1) diode laser (405 nm) exciting DAPI; (2) Ar laser (three lines: 458 nm, 488 nm and 514 nm), exciting both FITC (green) and Cy3/PI (red); (3) He-Ne laser green (543 nm), exciting Cy3, and (4) He-Ne lase red (633) exciting Cy5.

When cells were double transfected, so that they were stained simultaneously with both dyes (FITC-Cy3) a sequential scan was performed, using lasers 1 and 2 one after the other, to avoid cross-talk. Images were acquired with a 63x objective, using the built-in software from Leica.

2.5. Protein purification

2.5.1. Expression and purification of recombinant proteins

We used the pET11d expression vector (Novagen) where the proteins to be purified harbor a 6x-histidine tag at their N-termini (T7 system from Novagen). *E. coli* BL21(DE3) pLys S (Novagen) were transfomed with the pET11d vector and selected on LB/ampicillin/chloramphenicol plates. The cells were harvested by centrifugation for 5 minutes at 5000 *g* and the pellet resuspended in 20 ml lysis buffer (500 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.3, 5 mM β-Mercaptoethanol, 0.1% IGEPAL CA-630 (NP-40), 2 μg/ml Leupeptin, 1 μg/ml

Pepstatin A, 20 μg/ml Benzamidine and 1 mM Phenylmethylsulfonyl fluoride). All remaining steps were performed at 4 °C with pre-chilled buffers. Samples were sonicated on ice (Branson digital sonifier): amplitude 20%, for 3 minutes; on 0.2 sec, off 0.8 sec (i.e. total elapsed time 15 minutes) and spun for 30 minutes at 39,000 *g*. The pellet was discarded and imidazole added to the supernatant to a final concentration of 5 mM before loading onto a pre-equilibrated Ni^{2+}-column (0.8 ml, Amersham). The column was washed with BC400 (20 mM Tris (pH 7.3 at 25 °C), 20% glycerol, 1 mM PMSF, 5 mM DTT, 1 mM EDTA pH 8.0, 400 mM KCl) including 10 mM imidazole. The recombinant proteins were eluted with 100 mM imidazole in BC400 and then loaded onto a 0.5 ml Heparin Sepharose column (Amersham) and washed with 30 ml BC200 (as BC400, except 200 mM KCl). The recombinant protein was then eluted with BC600 (as BC400, except 600 mM KCl) and the protein total concentration measured and adjusted to at least 500 ng/μl with BSA (Roche Diagnostics GmbH). Hepes-KOH (pH 7.9 at 25 °C) was added to a final concentration of 50 mM, and aliquots were frozen in liquid nitrogen and stored at -80°C. The human TBP-pet11d vector is originally from the lab of B Roeder and the human NC2 pet11d exists both as two single subunit-vectors, or a bicistronic co-expression vector. NC2 truncation mutants were constructed using PCR primers harboring a stop codon at the desired place, and the sequences of the final vectors were confirmed by sequencing.

2.5.2. Preparation of whole cell extracts, WCE

Cells were collected, washed once with ice-cold PBS, resuspended in ca. 1ml ice-cold PBS and transferred in a 1.5 or 2 ml eppendorf. Cells were centrifuged 4 min at 4000 rpm at 4 °C, the supernatant was removed carefully and the cell pellet was gently resuspended in PBS at a final concentration of $2.3x10^7$ cells/ml. An equal volume of 2x cell culture lysis buffer, supplemented with fresh protease and phosphatase inhibitors, was added. After gentle vortexing, cells were kept in ice for 15 min, frozen in a dry ice-ethanol bath and thawed in a water bath at 37 °C. The cell debris was spun down at high speed (13200 rpm, 10 min at 4°C) and the supernatant was collected. Protein concentration was determined by the Bradford assay. The total protein extract was stored at -80°C. For western blot analysis, 3-5 *μ*l of extracts were used.

Interphase and mitotic HeLa WCE

For the big prep of mitotic and interphase HeLa WCE used to immunopurify native NC2, 240x 15 cm plates were collected in three different experiments. Each time,

60x plates were used for preparing mitotic (M) cells and 20x for the interphase (I). This gave approximately the same amount of M and I cells, corresponding to 3-4x10^7 cells for each sample in each experiment. M cells were prepared as described above. At the moment of harvesting, plates were shacked and only the detached cells were collected.
Interphase cells were obtained discarding the mitotic cells by shaking off and collecting only the attached ones.

Jurkat WCE for 2D gel analysis
For the big prep of Jurkat WCE used for the 2D analysis, three spinner cultures were grown in RPMI Medium (Gibco). These cultures were: (i) asynchronous cells, (ii) asynchronous cells stimulated 30 min with PMA (0.05 μM), and (iii) mitotic blocked cells. During culturing, cell density was kept between 3-6x10^6 cells/ml. For each population, 3.9-4.0x10^{10} cells (6.5 l) were harvested, and then centrifuged at 500 *g* for 25 min with a G3 rotor. Cell lysate was prepared as described above.

2.5.3. HeLa Nuclear Extracts

All the steps were performed at 4°C and with ice-cold solutions.
HeLA suspension cells were washed with PBS, centrifuged for 10 min at 2500 rpm, and washed in HB-buffer. After centrifugation, cells were resuspended in a volume of HB-buffer corresponding to 4x the initial packed cell volume. Cells were incubated in ice for 10 min and then the swollen cells were dounce homogenized 15x with the pistle B. The cells membrane debris was separated from the nuclei by centrifugation for 15 min at 3900 rpm. Nuclei were resuspended with low salt buffer (0.02 M KCl) in 1/2x of the volume corresponding to the nuclei pellet and then the same amount of high salt buffer (1.6 M KCl) was added drop by drop in 30 min. The nuclear extracts were centrifuged for 30 min at 14000 rpm, the supernatant collected, snap frozen in liquid nitrogen and stored at -80°C.

2.5.4 Measurement of protein concentration

Total protein concentration was determined using the Bradford assay (Bio-Rad protein assay) by detecting absorbance at 595 nm. A standard curve was made using serial dilution of BSA (100-250-500-750-1000 μg/ml) and protein concentration of the sample was calculated according to the standard curve.

2.5.5. Coupling of antibody to the beads

All centrifugation steps were done at 2000 rpm for 2 min. 1-2 mg of rat monoclonal antibody (hybridoma supernatant, ca. 50 μg/ml) were incubated with 1 ml of wet

Protein G-Sepharose (Amersham) over night in gentle rocking in the cold room. Beads were washed three times with PBS and three times with 0.2 M sodium borate (pH 9). After resuspension in 10 ml (10 volumes) of 0.2 M sodium borate (pH 9.0), 52 mg of dimethyl pimelidate (DMP) were added. Beads were incubated for 30 minutes at room temperature with gentle mixing. The reaction was stopped by washing the beads twice with 0.2 M ethanolamine (pH 8.0), followed by 2 h incubation with the same solution in gentle rocking at room temperature. At the end, beads were washed twice with PBS and resuspended in PBS containing 0.02% Sodium Azide, and stored at 4° C.

2.5.6. Mapping of the epitope recognized by the NC2α 4G7 antibody

Both NC2α monoclonal ab (4G7, 6G8) recognize a sequence in the C-terminus of the protein, but it was not known which amino acids correspond to the epitope. Knowledge of this sequence allows elution of the NC2 bound to the antibody by mean of a synthetic peptide mapping this epitope instead of using SDS buffer or pH elution (which lead to protein denaturation). Protein eluted with the peptide can therefore be readily used for *in vitro* assay. Three partially overlapping peptides (20-21 aa long) were tested:

159: 159-PPQASHAPSAHFQSPPTPFLP-178
171: 171-SPPTPFLPFASTLPLPPAPP-190
186: 86-PPAPPGPSAPDEEDEEDYDS-205

The peptides were eluted in 40 mM Hepes 7.6 to a final concentration of 3.5 mg/ml. The peptides were spotted in nitrocellulose membrane at three different concentrations (10, 100, 1000 ng). As a control, an unrelated peptide was also spotted. Then, the membrane was immunoblotted for both ab. The 159 peptide was weakly recognized by the 4G7 ab and strongly by the 6G8 ab. The opposite was observed for the 171 peptide, while neither ab recognized the 186 peptide. The ability of 159 and 171 to elute some recombinant NC2 bound to protein G ab was tested. Only the 171 peptide eluted 90% of the protein, while 159 did not. Thus, 171 was used for all peptide elutions, with the following conditions:
1-1.5 mg/ml peptide in BC100 or 150 / 0.1% NP40. Beads were eluted twice, each time with one column volume, rotating for 30 min at RT.

2.5.7. Immunoprecipitation

All steps were performed at 4°C and all buffers were supplemented with fresh DTT (1mM), protease inhibitors (200 mM PMSF, 100 mM Benzamidine, 2 μg/ml

Leupeptin, 2 μg/ml Aprotinin, 0.2 μg/ml Pepstatin A) and phosphatase inhibitors (1 mM NaF, 100 mM Vanadate). Each step was separated by a centrifugation at 2500 rpm for 2 min. To deplete NC2α, 1ml of NE or WCE were incubated with either 200 μl or 150 μl of ab-coupled beads, respectively. To reduce unspecific binding, the extracts were precleared incubating them with Protein G-Sepharose coupled with an antibody of the same isotype of NC2α for two hours in gentle rocking (IgG1, CAD9, kindly provided by E. Kremmer). Extracts were then incubated with Protein G-Sepharose coupled with the specific NC2α ab for at least 2 h, rotating at 4 °C. Beads were washed 5x with BC500/0.1% NP40, followed by 3x washes with BC150/0.1% NP40. Proteins were eluted twice from the ab either with 2x SDS buffer (1 min, 95 °C), or with the peptide corresponding to the epitope (see above), each time with one column volume.

2.6. Protein analysis

2.6.1. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

15% separating gel was cast using 30% (w/v) Acrylamide (GelA), 2% (w/v) Bisacrylamid (GelB) (Roth). For electrophoresis, protein samples were mixed 1:6 with 6xloading buffer, heat denatured for 2 min at 95 °C and loaded onto the gel. Proteins were separated by applying a current of 20 mA until the dye front had reached the end of the gel. Unstained marker proteins (Bio-Rad) were run in parallel. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue G250, silver staining or subjected to Western blotting (see below).

6X loading buffer:

0.35 M Tris-HCl (pH 6.8 RT)
10% (w/v) SDS
30% (v/v) Glycerol
9.3% (w/v) DTT
0.12 mg/ml Bromphenolblue

Reagents	Separating gel 15% (170:1) (30ml)	Stacking gel (10ml)
H_2O	6 ml	6.2 ml
GelA	20 ml	1.7 ml
GelB	1.3 ml	0.7 ml

1.5 M Tris-HCl (pH 8.8 RT)	7.5 ml	
1 M Tris-HCl (pH 6.8 RT)		1.25 ml
10% (w/v) SDS	0.3 ml	0.1 ml
10% (w/v) APS	100 µl	50 µl
TEMED	30 µl	25 µl

SDS-PAGE Protein-Standards for low molecular weight proteins (Bio-Rad)

Proteins	MW (kD)
Phosphorylase B	97 kD
BSA	66 kD
Ovalbumin	45 kD
Carboanhydrase	31 kD
Trypsin Inhibitor	21.5 kD
Lysozyme	14.4 kD

2.6.2. Coomassie staining of polyacrylamide gels

For Coomassie staining of polyacrylamide gels, the gels were incubated minimum 2 h on a slowly rocking platform with Staining solution (40% Methanol, 10% Glacial Acetic acid, 0.25% Coomassie Brilliant Blue R-250). To visualize the proteins the gels were incubated overnight in destaining solution (50% Methanol, 10% Glacial Acetic acid). For drying the gels were soaked in dH_2O, placed between cellophane film and dried at RT.

2.6.3. Silver staining of polyacrylamide gels

Silver stained of the gel was performed with the PlusOne-kit (Amersham) according to the manufacture instruction. The gels were fixed in 2.5% acetic acid, 2.5% methanol, rinsed 2x with dH_2O and shaken in dH_2O for 2 h to overnight. Sensitization was with 0.02% sodium thiosulphate for 2 min, followed by rinsing 2x30 sec with dH_2O. The gels were then incubated 30 min in 0.1% $AgNO_3$, rinsed 2x30 sec with dH_2O and developed with 0.01% formaldehyde, 2% sodium carbonate. When a sufficient degree of staining was reached the developing solution was poured out and the process was stopped with several changes of 1% acetic acid.

2.6.4. Western blotting (immunoblotting)

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Bio-Rad) using semi-dry blot transfer (Bio-Rad or Amersham).

For protein transfer, the gel was sandwiched between gel-sized Whatmann 3MM papers soaked in transfer buffer (20% methanol in 1xTris/glycine buffer) and run for 45 min at 15V.

After transfer, nitrocellulose filters were incubated for 1 h in a Blocking solution (TBS containing 6% dried milk) in order toreduce the unspecific background. The membrane was incubated for 1 h in appropriate dilution of the primary antibodies, directed against the protein of interest. The membrane was washed 3 times for at least 10 min in TBST and incubated for an additional 45 min with the appropriate secondary antibody conjugated to horseradish peroxidase or alkaline phosphatase. After 3 washes in PBST, antigen-antibody complexes were detected using the enhanced chemiluminescence detection system (NEN, Boston, MA or Amersham Biosciences), according to the manufacturer's instructions and exposed on Biomax-MR film (Eastman Kodak, Rochester, NY).

Primary antibody	Dilution	Secondary antibody	Dilution
Rat monoclonal NC2α-4G7	1:10	-rat-HRP (Promega)	1:4000
		-rat-AP (Promega)	1:3000
Rabbit polyclonal NC2β	1:500	-rabbit-HRP (Promega)	1:6000
		-rabbit-AP (Promega)	1:3000
Rabbit TBP (Santa Cruz, Cat. No. sc-273)	1:1000	-rabbit-HRP (Promega)	1:6000

2.6.5. 2D Polyacrilammide gel electrophoresis

In 2D electrophoresis gels, proteins are separated according to their charge (*i.e*. their isoelectric point, pI), and on the basis of their molecular weight. First, proteins are run using an electric field, so that they migrate until they reach their pI (charge = 0); then, the immobilized pH gradient (IPG) strip is rotated 90° and the proteins are separated by size in a standard SDS-PAGE without stacking gel.

Native NC2 was immunopurified from three different Jurkat WCE, each made from 3-3.5x10^{10} cells (asynchronous -/+ PMA and M-blocked). As a control, the asynchronous WCE were also incubated with a column with bound an antibody

of the same isotype than the NC2α ab that does not recognize any protein in the WCE. Proteins eluted from this column represented the control for background.
Proteins were eluted from the antibody-coupled beads with 2 ml urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, bromophenolblue at RT). To minimize salt concentration, the elutions were concentrated to 20 μl with a centrifugal concentrator (Vivaspin 2, MWCO 10 kD; VIVASCIENCE), and then 100 μl of urea buffer were added. To this solution, 0.5% IPG and 1% DTT were added, samples were shaken at 20 °C for 30 min, and then stored at -80 °C.
2D gels were performed with a standard protocol, according to the method of Gorg et al. (1988). Protein samples were applied to IPG strips, 18 cm long and with a pH gradient between pH 4-7. Proteins were focused using the IPGPhor Amersham). After isoelectric focusing, proteins in the IGP strips were separated by SDS-PAGE (T = 13%, C = 3%) and either silver stained or blotted to PVDF membrane (Amersham) for immunoblot analysis.

2.6.6. Computer densitometry analysis

2D gels made from the specific samples (Asynchronous -/+ PMA and M) were compared with the control gel (c Async). The digitized images from these gels were compared to identify the proteins present exclusively in the specific samples.

2.6.7. Mass Spectrometry

Spot identification was done by peptide mass fingerprinting. Mass spectrometry can identify molecules based on very accurate measurements of their mass/charge (m/z) ratio and identify post-translational modification based on the detection of mass changes. Only peptides with mass comprised in a certain range (300-2500 D) can be identified. Thus, proteins cut out from the gels were digested either with Glu C (NC2β spots) or trypsin (all the others). Peptides were processed for the MALDI-TOF analysis according to the standard method.

The 2D gels and computer densitometry analysis presented in this work were performed at the Toplab AG (Martinsried). Two different Mass Spectrometry analysis were performed, in the Toplab AG and by Gerhard Mittler (at the Mathias Mann laboratory, Odense, Denmark).

2.7. Analysis of protein-DNA interaction

2.7.1. Electromobility shift assay (EMSA)

35 bp oligonucleotides including a 4 bp 5'overhang were obtained from MWG-Biotech AG. Their concentrations were controlled using a spectrophotometer. Oligos were annealed in buffer (200 mM NaCl, 10 mM Tris-HCl pH 7.3, 1 mM $MgCl_2$) by heating to 95°C for 3 minutes and cooling gradually to room temperature. The annealed oligos were stored at 4 °C and labelled with ^{32}P-αdCTP (Amersham) by Klenow fill-in by standard procedures. The 35 bp sequences used were as follows (TATA-sequence underlined):

ML_up: 5'-CCTGAAGGGGGGCTATAAAAGGGGGTGGGGGCGCG-3'

ML_down: 5'-CGCGCCCCCACCCCCCTTTTATAGCCCCCCTT-3'

Labelling DNA

The short 35 bp oligo containing the Adenovirus Major Late Promoter (35 bp AdMLP oligo) was labeled by filling-in of recessed 3'-termini of double-stranded DNA, mixing:

Reagent	Amount
DNA	10 pmol
Buffer Klenow	2 μl
3 dNTP-mix (3.3 mM)	2.5 μl
Water to a total volume of 15 μl	
water	to a total volume of 15 μl
add α-^{32}P-dNTP (3000 Ci/mmol)	4 μl
Klenow fragment (2 u/μl)	1 μl
Incubate the mixture at 30°C for 15 minutes	
add 30 μl TE	
Purify the labeled fragment with MicroSpin column G-25, according to the manufacturer's instructions (Amersham)	
Dilute in TE (100 fmol/μl) and keep at 4°C (up to 3 weeks).	

The long DNA fragment (217 bp) containing the Adenovirus Major Late Promoter is derived from the pB2-MLP vector (kindly provided by M. Timmers). The DNA fragment was obtained by standard PCR of the SmaI fragment from the pB2-MLP vector. Two amplification reactions were performed, each with one of the two primers (forward or reverse) labeled in 5' with radioactivity. In this way, the resulting DNA fragment was marked only in one of the two strands and used for both EMSA and

DNA footprint. After amplification, the desired band was separated from unspecific products in agarose gel. The labeling of primers at 5' was done with Polynucleotide kinase. Sequences of the primers are:
Sma-1: 5'-GGAGGCCTTCGCG-3'
Sma-2: 5'-GGGCAGCTGGATATC-3'

Reagent	Amount
primer	10 pmol
Buffer T4 Polynucleotide kinase	2 μl
Water to a total volume of 14 μl	
add γ-^{32}P-ATP (3000 Ci/mmol)	5 μl
T4 Polynucleotide kinase (10 u/μl)	1 μl
Incubate the mixture at 37°C for 30 minutes	
add 30 μl TE	
Purify the labeled fragment with MicroSpin column G-25, according to the manufacturer's instructions (Amersham)	
Dilute in TE (100 fmol/μl) and keep at 4°C (up to 3 weeks).	

The Nucleosome template DNA was kindly provided by G. Langst. Nucleosomes were assembled on a 248 bp rDNA fragment using purified histones, according to Langst and Becker (Langst and Becker 2001).

The binding reactions for EMSA were carried out in a final volume of 20 μl, with 4 mM $MgCl_2$, 25 mM Hepes-KOH pH 8.2, 0.4 mg/ml BSA, 5 mM DTT, 0.5 mM PMSF 50-200 fmol labelled DNA. The final concentration of glycerol was kept between 7-10% and the final concentration of KCl between 70-90 mM. Protein amounts are given in figure legends. Binding reactions were incubated 30 minutes at 27 °C before loading on 5% acrylamide gels (acrylamide:bisacrylamide 50:1) and run at 120 V in TGE buffer. Band intensity was quantified using a phosphoimager (Packard Instantimager). When using long DNA, 0.5% TBE buffer was used instead.

The amounts of protein used for EMSA refer to the active protein concentrations. NC2 was kept at limiting concentration, while TBP was in excess, and DNA was titrated until no increase in complex formation was observed. Under these conditions, all NC2 proteins capable of binding participate in complex formation. This is defined as the active concentration of the protein, and is expressed as fmol of bound DNA.

2.7.2. Footprint

The binding reactions for footprint were carried out as follow: 100 fmol of end-labeled DNA fragment (see above) were incubated with 25 ng of yTBP (350 fmol) with NC2 in concentration ranging from 50-2000 fmol (3.5-140 ng recombinant NC2). Reactions were incubated for 30 min at 28 °C in 20 μl volume, with 20 mM Hepes-KOH (pH 8.2), 60-70 mM KCl, 2.5 mM Sodiumphosphate (pH 7.6), 5 mM $MgCl_2$, 6-10% glycerol, 5 mM DTT, 1 mM PMSF, 0.05% NP40.

1-10 ng DNAseI in 5 μl buffer 2 (5 mM DTT, 5 mM $CaCl_2$, 100 ng dGdC, 50 mM KCl, 10 mM Hepes-KOH pH 8.2 and 2.5 μg BSA) was added to the binding reaction and incubated for 20 to 30 sec.

The reaction was stopped, precipitated in Phenol-chloroform and run on 7.5% denaturing gel with 0.7x TBA.

LIST OF ABBREVIATIONS

ab	antibody
APS	ammonium persulphate
Ar	Argon
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
Cat. No.	catalogue number
CoIP	co-immunoprecipitation
dH_2O	distilled water
DNTP	deoxynucleoside triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
FBS = FCS	foetal bovine serum = foetal calf serum
Fig.	figure
FL	full length
g	gram
GTP	guanosine 5'-triphosphate
He	Helion
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
His	hexahistidine tag
h	hour
kD	kilo Dalton
l	liter
Ig	Immunoglobulin Tag
M	molar (mol/l)
Mab	Monoclonal antibody
min	minute
MW	molecular weight
NC2	Negative Cofactor 2
Ne	Neon
OD	optical density

PCR	polymerase chain reaction
PFA	paraformaldehyde
PI	propidium iodide
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecylsulphate
SDS PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
sec	seconds
SP	streptavidin
TBP	TATA Binding Protein
Tris	Tris(hydroxymethyl)aminomethane
V	volt(s)
W	watt(s)
wt	wild–type

REFERENCES

Akoulitchev, S. and D. Reinberg. 1998. The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. *Genes Dev* **12**: 3541-50.

Albright, S.R. and R. Tjian. 2000. TAFs revisited: more data reveal new twists and confirm old ideas. *Gene* **242**: 1-13.

Allfrey, V.G., R. Faulkner, and A.E. Mirsky. 1964. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. *Proc Natl Acad Sci U S A* **51**: 786-94.

Andersen, J.S., C.E. Lyon, A.H. Fox, A.K. Leung, Y.W. Lam, H. Steen, M. Mann, and A.I. Lamond. 2002. Directed proteomic analysis of the human nucleolus. *Curr Biol* **12**: 1-11.

Andrau, J.C., C.J. Van Oevelen, H.A. Van Teeffelen, P.A. Weil, F.C. Holstege, and H.T. Timmers. 2002. Mot1p is essential for TBP recruitment to selected promoters during *in vivo* gene activation. *Embo J* **21**: 5173-83.

Aoyagi, N. and D.A. Wassarman. 2000. Genes encoding Drosophila melanogaster RNA polymerase II general transcription factors: diversity in TFIIA and TFIID components contributes to gene-specific transcriptional regulation. *J Cell Biol* **150**: F45-50.

Apone, L.M., C.M. Virbasius, J.C. Reese, and M.R. Green. 1996. Yeast TAF(II)90 is required for cell-cycle progression through G2/M but not for general transcription activation. *Genes Dev* **10**: 2368-80.

Appella, E. and C.W. Anderson. 2000. Signaling to p53: breaking the posttranslational modification code. *Pathol Biol (Paris)* **48**: 227-45.

Asturias, F.J., Y.W. Jiang, L.C. Myers, C.M. Gustafsson, and R.D. Kornberg. 1999. Conserved structures of mediator and RNA polymerase II holoenzyme. *Science* **283**: 985-7.

Auble, D.T., K.E. Hansen, C.G. Mueller, W.S. Lane, J. Thorner, and S. Hahn. 1994. Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. *Genes Dev* **8**: 1920-34.

Bannister, A.J. and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**: 641-3.

Bannister, A.J., R. Schneider, and T. Kouzarides. 2002. Histone methylation: dynamic or static? *Cell* **109**: 801-6.

Becker, P.B. and W. Horz. 2002. ATP-dependent nucleosome remodeling. *Annu*

Rev Biochem **71**: 247-73.

Bell, B., E. Scheer, and L. Tora. 2001. Identification of hTAF(II)80 delta links apoptotic signaling pathways to transcription factor TFIID function. *Mol Cell* **8**: 591-600.

Bengal, E., O. Flores, A. Krauskopf, D. Reinberg, and Y. Aloni. 1991. Role of the mammalian transcription factors IIF, IIS, and IIX during elongation by RNA polymerase II. *Mol Cell Biol* **11**: 1195-206.

Bentley, D. 2002. The mRNA assembly line: transcription and processing machines in the same factory. *Curr Opin Cell Biol* **14**: 336-42.

Berger, S.L. 2001. Molecular biology. The histone modification circus. *Science* **292**: 64-5.

-.2002. Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* **12**: 142-8.

Biggs, W.H., 3rd, J. Meisenhelder, T. Hunter, W.K. Cavenee, and K.C. Arden. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96**: 7421-6.

Bleichenbacher, M., S. Tan, and T.J. Richmond. 2003. Novel interactions between the components of human and yeast TFIIA/TBP/DNA complexes. *J Mol Biol* **332**: 783-93.

Bogerd, H.P., R.A. Fridell, R.E. Benson, J. Hua, and B.R. Cullen. 1996. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol Cell Biol* **16**: 4207-14.

Borggrefe, T., R. Davis, A. Bareket-Samish, and R.D. Kornberg. 2001. Quantitation of the RNA polymerase II transcription machinery in yeast. *J Biol Chem* **276**: 47150-3.

Boube, M., L. Joulia, D.L. Cribbs, and H.M. Bourbon. 2002. Evidence for a mediator of RNA polymerase II transcriptional regulation conserved from yeast to man. *Cell* **110**: 143-51.

Boyle, W.J., T. Smeal, L.H. Defize, P. Angel, J.R. Woodgett, M. Karin, and T. Hunter. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**: 573-84.

Bradbury, E.M. 1992. Reversible histone modifications and the chromosome cell cycle. *Bioessays* **14**: 9-16.

Bradbury, E.M., R.J. Inglis, H.R. Matthews, and T.A. Langan. 1974. Molecular basis of control of mitotic cell division in eukaryotes. *Nature* **249**: 553-6.

Brou, C., S. Chaudhary, I. Davidson, Y. Lutz, J. Wu, J.M. Egly, L. Tora, and P.

Chambon. 1993. Distinct TFIID complexes mediate the effect of different transcriptional activators. *Embo J* **12**: 489-99.

Brownell, J.E., J. Zhou, T. Ranalli, R. Kobayashi, D.G. Edmondson, S.Y. Roth, and C.D. Allis. 1996. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**: 843-51.

Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**: 857-68.

Brunet, A., S.R. Datta, and M.E. Greenberg. 2001a. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* **11**: 297-305.

Brunet, A., J. Park, H. Tran, L.S. Hu, B.A. Hemmings, and M.E. Greenberg. 2001b. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* **21**: 952-65.

Buratowski, S., S. Hahn, L. Guarente, and P.A. Sharp. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56**: 549-61.

Buratowski, S., S. Hahn, P.A. Sharp, and L. Guarente. 1988. Function of a yeast TATA element-binding protein in a mammalian transcription system. *Nature* **334**: 37-42.

Burgess-Beusse, B., C. Farrell, M. Gaszner, M. Litt, V. Mutskov, F. Recillas-Targa, M. Simpson, A. West, and G. Felsenfeld. 2002. The insulation of genes from external enhancers and silencing chromatin. *Proc Natl Acad Sci U S A* **99 Suppl 4**: 16433-7.

Burke, T.W. and J.T. Kadonaga. 1997. The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila. *Genes Dev* **11**: 3020-31.

Burley, S.K. and R.G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu Rev Biochem* **65**: 769-99.

Busso, D., A. Keriel, B. Sandrock, A. Poterszman, O. Gileadi, and J.M. Egly. 2000. Distinct regions of MAT1 regulate cdk7 kinase and TFIIH transcription activities. *J Biol Chem* **275**: 22815-23.

Butler, J.E. and J.T. Kadonaga. 2002. The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev* **16**: 2583-92.

Caelles, C., H. Hennemann, and M. Karin. 1995. M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. *Mol Cell Biol* **15**: 6694-701.

Camporeale, G., E.E. Shubert, G. Sarath, R. Cerny, and J. Zempleni. 2004. K8 and

K12 are biotinylated in human histone H4. *Eur J Biochem* **271**: 2257-63.

Cang, Y., D.T. Auble, and G. Prelich. 1999. A new regulatory domain on the TATA-binding protein. *Embo J* **18**: 6662-71.

Cang, Y. and G. Prelich. 2002. Direct stimulation of transcription by negative cofactor 2 (NC2) through TATA-binding protein (TBP). *Proc Natl Acad Sci U S A* **99**: 12727-32.

Castano, E., P. Gross, Z. Wang, R.G. Roeder, and T. Oelgeschlager. 2000. The C-terminal domain-phosphorylated IIO form of RNA polymerase II is associated with the transcription repressor NC2 (Dr1/DRAP1) and is required for transcription activation in human nuclear extracts. *Proc Natl Acad Sci U S A* **97**: 7184-9.

Chambon, P. 1978. Summary: the molecular biology of the eukaryotic genome is coming of age. *Cold Spring Harb Symp Quant Biol* **42 Pt 2**: 1209-34.

Chang, M. and J.A. Jaehning. 1997. A multiplicity of mediators: alternative forms of transcription complexes communicate with transcriptional regulators. *Nucleic Acids Res* **25**: 4861-5.

Chao, D.M., E.L. Gadbois, P.J. Murray, S.F. Anderson, M.S. Sonu, J.D. Parvin, and R.A. Young. 1996. A mammalian SRB protein associated with an RNA polymerase II holoenzyme. *Nature* **380**: 82-5.

Chen, B.S. and M. Hampsey. 2004. Functional interaction between TFIIB and the Rpb2 subunit of RNA polymerase II: implications for the mechanism of transcription initiation. *Mol Cell Biol* **24**: 3983-91.

Chen, C.A. and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6**: 632-8.

Chen, D., C.S. Hinkley, R.W. Henry, and S. Huang. 2002. TBP dynamics in living human cells: constitutive association of TBP with mitotic chromosomes. *Mol Biol Cell* **13**: 276-84.

Chen, H.Y., J.M. Sun, Y. Zhang, J.R. Davie, and M.L. Meistrich. 1998. Ubiquitination of histone H3 in elongating spermatids of rat testes. *J Biol Chem* **273**: 13165-9.

Chen, W. and K. Struhl. 1988. Saturation mutagenesis of a yeast his3 “TATA element”: genetic evidence for a specific TATA-binding protein. *Proc Natl Acad Sci U S A* **85**: 2691-5.

Chicca, J.J., 2nd, D.T. Auble, and B.F. Pugh. 1998. Cloning and biochemical characterization of TAF-172, a human homolog of yeast Mot1. *Mol Cell Biol* **18**: 1701-10.

Chitikila, C., K.L. Huisinga, J.D. Irvin, A.D. Basehoar, and B.F. Pugh. 2002. Interplay

of TBP inhibitors in global transcriptional control. *Mol Cell* **10**: 871-82.

Cho, E.J., M.S. Kobor, M. Kim, J. Greenblatt, and S. Buratowski. 2001. Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-termial domain. *Genes Dev* **15**: 3319-3329.

Christova, R. and T. Oelgeschlager. 2002. Association of human TFIID-promoter complexes with silenced mitotic chromatin *in vivo*. *Nat Cell Biol* **4**: 79-82.

Cisek, L.J. and J.L. Corden. 1989. Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. *Nature* **339**: 679-84.

Coin, F. and J.M. Egly. 1998. Ten years of TFIIH. *Cold Spring Harb Symp Quant Biol* **63**: 105-10.

Cokol, M., R. Nair, and B. Rost. 2000. Finding nuclear localization signals. *EMBO Rep* **1**: 411-5.

Coleman, T.R. and W.G. Dunphy. 1994. Cdc2 regulatory factors. *Curr Opin Cell Biol* **6**: 877-82.

Collart, M.A. 1996. The NOT, SPT3, and MOT1 genes functionally interact to regulate transcription at core promoters. *Mol Cell Biol* **16**: 6668-76.

Conaway, R.C. and J.W. Conaway. 1993. General initiation factors for RNA polymerase II. *Annu Rev Biochem* **62**: 161-90.

Corona, D.F., A. Eberharter, A. Budde, R. Deuring, S. Ferrari, P. Varga-Weisz, M. Wilm, J. Tamkun, and P.B. Becker. 2000. Two histone fold proteins, CHRAC-14 and CHRAC-16, are developmentally regulated subunits of chromatin accessibility complex (CHRAC). *Embo J* **19**: 3049-59.

Cosma, M.P., T. Tanaka, and K. Nasmyth. 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* **97**: 299-311.

Courey, A.J. and S. Jia. 2001. Transcriptional repression: the long and the short of it. *Genes Dev* **15**: 2786-96.

Cox, J.M., M.M. Hayward, J.F. Sanchez, L.D. Gegnas, S. van der Zee, J.H. Dennis, P.B. Sigler, and A. Schepartz. 1997. Bidirectional binding of the TATA box binding protein to the TATA box. *Proc Natl Acad Sci U S A* **94**: 13475-80.

Cramer, P., D.A. Bushnell, J. Fu, A.L. Gnatt, B. Maier-Davis, N.E. Thompson, R.R. Burgess, A.M. Edwards, P.R. David, and R.D. Kornberg. 2000. Architecture of RNA polymerase II and implications for the transcription mechanism. *Science* **288**: 640-9.

Cramer, P., D.A. Bushnell, and R.D. Kornberg. 2001. Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* **292**: 1863-76.

Creton, S., J.Q. Svejstrup, and M.A. Collart. 2002. The NC2 alpha and beta subunits

play different roles *in vivo*. *Genes Dev* **16**: 3265-76.

Dahmus, M.E. 1996. Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem* **271**: 19009-12.

Dantonel, J.C., J.M. Wurtz, O. Poch, D. Moras, and L. Tora. 1999. The TBP-like factor: an alternative transcription factor in metazoa? *Trends Biochem Sci* **24**: 335-9.

Dasgupta, A., R.P. Darst, K.J. Martin, C.A. Afshari, and D.T. Auble. 2002. Mot1 activates and represses transcription by direct, ATPase-dependent mechanisms. *Proc Natl Acad Sci U S A* **99**: 2666-71.

Davis, J.L., R. Kunisawa, and J. Thorner. 1992. A presumptive helicase (MOT1 gene product) affects gene expression and is required for viability in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* **12**: 1879-92.

Davis, L.I. 1995. The nuclear pore complex. *Annu Rev Biochem* **64**: 865-96.

De Cesare, D., G.M. Fimia, and P. Sassone-Corsi. 1999. Signaling routes to CREM and CREB: plasticity in transcriptional activation. *Trends Biochem Sci* **24**: 281-5.

DeJong, J. and R.G. Roeder. 1993. A single cDNA, hTFIIA/alpha, encodes both the p35 and p19 subunits of human TFIIA. *Genes Dev* **7**: 2220-34.

Denko, N., K. Wernke-Dollries, A.B. Johnson, E. Hammond, C.M. Chiang, and M.C. Barton. 2003. Hypoxia actively represses transcription by inducing negative cofactor 2 (Dr1/DrAP1) and blocking preinitiation complex assembly. *J Biol Chem* **278**: 5744-9.

Dhalluin, C., J.E. Carlson, L. Zeng, C. He, A.K. Aggarwal, and M.M. Zhou. 1999. Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**: 491-6.

Dikstein, R., S. Ruppert, and R. Tjian. 1996. TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. *Cell* **84**: 781-90.

Dotson, M.R., C.X. Yuan, R.G. Roeder, L.C. Myers, C.M. Gustafsson, Y.W. Jiang, Y. Li, R.D. Kornberg, and F.J. Asturias. 2000. Structural organization of yeast and mammalian mediator complexes. *Proc Natl Acad Sci U S A* **97**: 14307-10.

Dvir, A., R.C. Conaway, and J.W. Conaway. 1997. A role for TFIIH in controlling the activity of early RNA polymerase II elongation complexes. *Proc Natl Acad Sci U S A* **94**: 9006-10.

Earnshaw, W.C. and A.F. Pluta. 1994. Mitosis. *Bioessays* **16**: 639-43.

Eberharter, A. and P.B. Becker. 2002. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. *EMBO Rep* **3**: 224-9.

Elledge, S.J. and J.W. Harper. 1994. Cdk inhibitors: on the threshold of checkpoints and development. *Curr Opin Cell Biol* **6**: 847-52.

Estruch, F. and C.N. Cole. 2003. An early function during transcription for the yeast mRNA export factor Dbp5p/Rat8p suggested by its genetic and physical interactions with transcription factor IIH components. *Mol Biol Cell* **14**: 1664-76.

Feng, Q., H. Wang, H.H. Ng, H. Erdjument-Bromage, P. Tempst, K. Struhl, and Y. Zhang. 2002. Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. *Curr Biol* **12**: 1052-8.

Fink, K. and G. Turnock. 1977. Synthesis of transfer RNA during the synchronous nuclear division cycle in Physarum polycephalum. *Eur J Biochem* **80**: 93-6.

Flores, O., H. Lu, and D. Reinberg. 1992. Factors involved in specific transcription by mammalian RNA polymerase II. Identification and characterization of factor IIH. *J Biol Chem* **267**: 2786-93.

Flores, O., E. Maldonado, and D. Reinberg. 1989. Factors involved in specific transcription by mammalian RNA polymerase II. Factors IIE and IIF independently interact with RNA polymerase II. *J Biol Chem* **264**: 8913-21.

Fondell, J.D., H. Ge, and R.G. Roeder. 1996. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci U S A* **93**: 8329-33.

Forbes, D.J. 1992. Structure and function of the nuclear pore complex. *Annu Rev Cell Biol* **8**: 495-527.

Freiman, R.N., S.R. Albright, S. Zheng, W.C. Sha, R.E. Hammer, and R. Tjian. 2001. Requirement of tissue-selective TBP-associated factor TAFII105 in ovarian development. *Science* **293**: 2084-7.

Fuchs, S.Y., I. Tappin, and Z. Ronai. 2000. Stability of the ATF2 transcription factor is regulated by phosphorylation and dephosphorylation. *J Biol Chem* **275**: 12560-4.

Furter-Graves, E.M. and B.D. Hall. 1990. DNA sequence elements required for transcription initiation of the Schizosaccharomyces pombe ADH gene in Saccharomyces cerevisiae. *Mol Gen Genet* **223**: 407-16.

Gadbois, E.L., D.M. Chao, J.C. Reese, M.R. Green, and R.A. Young. 1997. Functional antagonism between RNA polymerase II holoenzyme and global negative regulator NC2 *in vivo*. *Proc Natl Acad Sci U S A* **94**: 3145-50.

Garcia-Bustos, J., J. Heitman, and M.N. Hall. 1991. Nuclear protein localization. *Biochim Biophys Acta* **1071**: 83-101.

Garcia-Ramirez, M., C. Rocchini, and J. Ausio. 1995. Modulation of chromatin folding by histone acetylation. *J Biol Chem* **270**: 17923-8.

Garrett, K.P., H. Serizawa, J.P. Hanley, J.N. Bradsher, A. Tsuboi, N. Arai, T. Yokota, K. Arai, R.C. Conaway, and J.W. Conaway. 1992. The carboxyl terminus of RAP30 is similar in sequence to region 4 of bacterial sigma factors and is required for function. *J Biol Chem* **267**: 23942-9.

Gautier, J., J. Minshull, M. Lohka, M. Glotzer, T. Hunt, and J.L. Maller. 1990. Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* **60**: 487-94.

Ge, H. and R. Roeder. 1994a. The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. *J Biol Chem* **269**: 17136-40.

Ge, H. and R.G. Roeder. 1994b. Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* **78**: 513-23.

Geiman, T.M. and K.D. Robertson. 2002. Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? *J Cell Biochem* **87**: 117-25.

Geisberg, J.V., F.C. Holstege, R.A. Young, and K. Struhl. 2001. Yeast NC2 associates with the RNA polymerase II preinitiation complex and selectively affects transcription *in vivo*. *Mol Cell Biol* **21**: 2736-42.

Geisberg, J.V., Z. Moqtaderi, L. Kuras, and K. Struhl. 2002. Mot1 associates with transcriptionally active promoters and inhibits association of NC2 in Saccharomyces cerevisiae. *Mol Cell Biol* **22**: 8122-34.

Ghosh, S., M.J. May, and E.B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**: 225-60.

Goodrich, J.A., T. Hoey, C.J. Thut, A. Admon, and R. Tjian. 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* **75**: 519-30.

Goppelt, A. and M. Meisterernst. 1996. Characterization of the basal inhibitor of class II transcription NC2 from Saccharomyces cerevisiae. *Nucleic Acids Res* **24**: 4450-5.

Goppelt, A., G. Stelzer, F. Lottspeich, and M. Meisterernst. 1996. A mechanism for repression of class II gene transcription through specific binding of NC2 to TBP-promoter complexes via heterodimeric histone fold domains. *Embo J* **15**: 3105-16.

Gorg, A., W. Postel, and S. Gunther. 1988. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **9**: 531-46.

Gorlich, D. 1997. Nuclear protein import. *Curr Opin Cell Biol* **9**: 412-9.

Gorlich, D. and R.A. Laskey. 1995. Roles of importin in nuclear protein import. *Cold Spring Harb Symp Quant Biol* **60**: 695-9.

Gorlich, D., F. Vogel, A.D. Mills, E. Hartmann, and R.A. Laskey. 1995. Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**: 246-8.

Gottesfeld, J.M. and D.J. Forbes. 1997. Mitotic repression of the transcriptional machinery. *Trends Biochem Sci* **22**: 197-202.

Gottesfeld, J.M., V.J. Wolf, T. Dang, D.J. Forbes, and P. Hartl. 1994. Mitotic repression of RNA polymerase III transcription *in vitro* mediated by phosphorylation of a TFIIIB component. *Science* **263**: 81-4.

Gregory, P.D., K. Wagner, and W. Horz. 2001. Histone acetylation and chromatin remodeling. *Exp Cell Res* **265**: 195-202.

Gu, W. and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**: 595-606.

Guarente, L. and E. Hoar. 1984. Upstream activation sites of the CYC1 gene of Saccharomyces cerevisiae are active when inverted but not when placed downstream of the “TATA box”. *Proc Natl Acad Sci U S A* **81**: 7860-4.

Ha, I., S. Roberts, E. Maldonado, X. Sun, L.U. Kim, M. Green, and D. Reinberg. 1993. Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. *Genes Dev* **7**: 1021-32.

Hahn, S. 2004. Structure and mechanism of the RNA polymerase II transcription machinery. *Nat Struct Mol Biol* **11**: 394-403.

Hake, S.B., A. Xiao, and C.D. Allis. 2004. Linking the epigenetic ‘language’ of covalent histone modifications to cancer. *Br J Cancer* **90**: 761-9.

Hans, F. and S. Dimitrov. 2001. Histone H3 phosphorylation and cell division. *Oncogene* **20**: 3021-7.

Harreman, M.T., T.M. Kline, H.G. Milford, M.B. Harben, A.E. Hodel, and A.H. Corbett. 2004. Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals. *J Biol Chem* **279**: 20613-21.

Hartl, P., J. Gottesfeld, and D.J. Forbes. 1993. Mitotic repression of transcription *in vitro*. *J Cell Biol* **120**: 613-24.

Hawkes, N.A. and S.G. Roberts. 1999. The role of human TFIIB in transcription start site selection *in vitro* and *in vivo*. *J Biol Chem* **274**: 14337-43.

Hernandez, N. 1993. TBP, a universal eukaryotic transcription factor? *Genes Dev* **7**: 1291-308.

Hershko, A. and A. Ciechanover. 1998. The ubiquitin system. *Annu Rev Biochem* **67**: 425-79.

Hershkovitz, M. and A.D. Riggs. 1995. Metaphase chromosome analysis by ligation-mediated PCR: heritable chromatin structure and a comparison of active and inactive X chromosomes. *Proc Natl Acad Sci U S A* **92**: 2379-83.

Hicks, G.R. and N.V. Raikhel. 1995. Protein import into the nucleus: an integrated view. *Annu Rev Cell Dev Biol* **11**: 155-88.

Hirano, T. and T.J. Mitchison. 1991. Cell cycle control of higher-order chromatin assembly around naked DNA *in vitro*. *J Cell Biol* **115**: 1479-89.

-. 1994. A heterodimeric coiled-coil protein required for mitotic chromosome condensation *in vitro*. *Cell* **79**: 449-58.

Hirose, Y. and J.L. Manley. 2000. RNA polymerase II and the integration of nuclear events. *Genes Dev* **14**: 1415-29.

Holmberg, C.I., S.E. Tran, J.E. Eriksson, and L. Sistonen. 2002. Multisite phosphorylation provides sophisticated regulation of transcription factors. *Trends Biochem Sci* **27**: 619-27.

Holstege, F.C., P.C. van der Vliet, and H.T. Timmers. 1996. Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. *Embo J* **15**: 1666-77.

Hood, J.K. and P.A. Silver. 1999. In or out? Regulating nuclear transport. *Curr Opin Cell Biol* **11**: 241-7.

Hunter, T. and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* **70**: 375-87.

Hurley, J.H., A.M. Dean, J.L. Sohl, D.E. Koshland, Jr., and R.M. Stroud. 1990. Regulation of an enzyme by phosphorylation at the active site. *Science* **249**: 1012-6.

Imbalzano, A.N., K.S. Zaret, and R.E. Kingston. 1994. Transcription factor (TF) IIB and TFIIA can independently increase the affinity of the TATA-binding protein for DNA. *J Biol Chem* **269**: 8280-6.

Imhof, A., X.J. Yang, V.V. Ogryzko, Y. Nakatani, A.P. Wolffe, and H. Ge. 1997. Acetylation of general transcription factors by histone acetyltransferases. *Curr Biol* **7**: 689-92.

Inostroza, J.A., F.H. Mermelstein, I. Ha, W.S. Lane, and D. Reinberg. 1992. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell* **70**: 477-89.

Iratni, R., Y.T. Yan, C. Chen, J. Ding, Y. Zhang, S.M. Price, D. Reinberg, and M.M. Shen. 2002. Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. *Science* **298**: 1996-9.

Jackson, S.P. 1992. Regulating transcription factor activity by phosphorylation. *Trends Cell Biol* **2**: 104-8.

Jacq, X., C. Brou, Y. Lutz, I. Davidson, P. Chambon, and L. Tora. 1994. Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell* **79**: 107-17.

Jans, D.A. 1995. The regulation of protein transport to the nucleus by phosphorylation. *Biochem J* **311 (Pt 3)**: 705-16.

Jans, D.A., C.Y. Xiao, and M.H. Lam. 2000. Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**: 532-44.

Jenuwein, T. and C.D. Allis. 2001. Translating the histone code. *Science* **293**: 1074-80.

Kadonaga, J.T. 2002. The DPE, a core promoter element for transcription by RNA polymerase II. *Exp Mol Med* **34**: 259-64.

Kaiser, K. and M. Meisterernst. 1996. The human general co-factors. *Trends Biochem Sci* **21**: 342-5.

Kalderon, D., W.D. Richardson, A.F. Markham, and A.E. Smith. 1984a. Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**: 33-8.

Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984b. A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499-509.

Kamada, K., F. Shu, H. Chen, S. Malik, G. Stelzer, R.G. Roeder, M. Meisterernst, and S.K. Burley. 2001. Crystal structure of negative cofactor 2 recognizing the TBP-DNA transcription complex. *Cell* **106**: 71-81.

Kang, J.J., D.T. Auble, J.A. Ranish, and S. Hahn. 1995. Analysis of the yeast transcription factor TFIIA: distinct functional regions and a polymerase II-specific role in basal and activated transcription. *Mol Cell Biol* **15**: 1234-43.

Kerem, B.S., R. Goitein, G. Diamond, H. Cedar, and M. Marcus. 1984. Mapping of DNAase I sensitive regions on mitotic chromosomes. *Cell* **38**: 493-9.

Kim, J.L., D.B. Nikolov, and S.K. Burley. 1993a. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365**: 520-7.

Kim, S., K. Cabane, M. Hampsey, and D. Reinberg. 2000. Genetic analysis of the YDR1-BUR6 repressor complex reveals an intricate balance among transcriptional regulatory proteins in yeast. *Mol Cell Biol* **20**: 2455-65.

Kim, S., J.G. Na, M. Hampsey, and D. Reinberg. 1997. The Dr1/DRAP1 heterodimer is a global repressor of transcription *in vivo*. *Proc Natl Acad Sci U S A* **94**: 820-5.

Kim, T.K., Y. Zhao, H. Ge, R. Bernstein, and R.G. Roeder. 1995. TATA-binding protein residues implicated in a functional interplay between negative cofactor NC2 (Dr1) and general factors TFIIA and TFIIB. *J Biol Chem* **270**: 10976-81.

Kim, U., X.F. Qin, S. Gong, S. Stevens, Y. Luo, M. Nussenzweig, and R.G. Roeder. 1996. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. *Nature* **383**: 542-7.

Kim, Y., J.H. Geiger, S. Hahn, and P.B. Sigler. 1993b. Crystal structure of a yeast TBP/TATA-box complex. *Nature* **365**: 512-20.

Kim, Y.J., S. Bjorklund, Y. Li, M.H. Sayre, and R.D. Kornberg. 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**: 599-608.

King, D.W. and M.L. Barnhisel. 1967. Synthesis of RNA in mammalian cells during mitosis and interphase. *J Cell Biol* **33**: 265-72.

King, R.W., P.K. Jackson, and M.W. Kirschner. 1994. Mitosis in transition. *Cell* **79**: 563-71.

Klejman, M.P., L.A. Pereira, H.J. van Zeeburg, S. Gilfillan, M. Meisterernst, and H.T. Timmers. 2004. NC2alpha interacts with BTAF1 and stimulates its ATP-dependent association with TATA-binding protein. *Mol Cell Biol* **24**: 10072-82.

Knauf, U., E.M. Newton, J. Kyriakis, and R.E. Kingston. 1996. Repression of human heat shock factor 1 activity at control temperature by phosphorylation. *Genes Dev* **10**: 2782-93.

Koleske, A.J. and R.A. Young. 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**: 466-9.

Konrad, C.G. 1963. Protein Synthesis and Rna Synthesis During Mitosis in Animal Cells. *J Cell Biol* **19**: 267-77.

Kornberg, R.D. 1977. Structure of chromatin. *Annu Rev Biochem* **46**: 931-54.

Kouzarides, T. 2002. Histone methylation in transcriptional control. *Curr Opin Genet Dev* **12**: 198-209.

Kretzschmar, M., K. Kaiser, F. Lottspeich, and M. Meisterernst. 1994. A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell* **78**: 525-34.

Kretzschmar, M., M. Meisterernst, and R.G. Roeder. 1993. Identification of human DNA topoisomerase I as a cofactor for activator-dependent transcription by RNA polymerase II. *Proc Natl Acad Sci U S A* **90**: 11508-12.

Kubota, S., T.D. Copeland, and R.J. Pomerantz. 1999. Nuclear and nucleolar targeting of human ribosomal protein S25: common features shared with HIV-1 regulatory proteins. *Oncogene* **18**: 1503-14.

Kuhn, E.J. and P.K. Geyer. 2003. Genomic insulators: connecting properties to mechanism. *Curr Opin Cell Biol* **15**: 259-65.

Kukimoto, I., S. Elderkin, M. Grimaldi, T. Oelgeschlager, and P.D. Varga-Weisz. 2004. The histone-fold protein complex CHRAC-15/17 enhances nucleosome sliding and assembly mediated by ACF. *Mol Cell* **13**: 265-77.

Kuo, M.T., B. Iyer, and R.J. Schwarz. 1982. Condensation of chromatin into chromosomes preserves an open configuration but alters the DNaseI hypersensitive cleavage sites of the transcribed gene. *Nucleic Acids Res* **10**: 4565-79.

la Cour, T., R. Gupta, K. Rapacki, K. Skriver, F.M. Poulsen, and S. Brunak. 2003. NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res* **31**: 393-6.

la Cour, T., L. Kiemer, A. Molgaard, R. Gupta, K. Skriver, and S. Brunak. 2004. Analysis and prediction of leucine-rich nuclear export signals. *Protein Eng Des Sel* **17**: 527-36.

Labbe, J.C., M.G. Lee, P. Nurse, A. Picard, and M. Doree. 1988. Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene cdc2+. *Nature* **335**: 251-4.

LaCasse, E.C. and Y.A. Lefebvre. 1995. Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. *Nucleic Acids Res* **23**: 1647-56.

Lachner, M. and T. Jenuwein. 2002. The many faces of histone lysine methylation. *Curr Opin Cell Biol* **14**: 286-98.

Lanford, R.E. and J.S. Butel. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**: 801-13.

Langst, G. and P.B. Becker. 2001. ISWI induces nucleosome sliding on nicked DNA. *Mol Cell* **8**: 1085-92.

Lee, D.K., M. Horikoshi, and R.G. Roeder. 1991. Interaction of TFIID in the minor groove of the TATA element. *Cell* **67**: 1241-50.

Lee, T.I., J.J. Wyrick, S.S. Koh, E.G. Jennings, E.L. Gadbois, and R.A. Young. 1998. Interplay of positive and negative regulators in transcription initiation by RNA polymerase II holoenzyme. *Mol Cell Biol* **18**: 4455-62.

Lee, T.I. and R.A. Young. 2000. Transcription of eukaryotic protein-coding genes. *Annu Rev Genet* **34**: 77-137.

Lemaire, M., J. Xie, M. Meisterernst, and M.A. Collart. 2000. The NC2 repressor is dispensable in yeast mutated for the Sin4p component of the holoenzyme and plays roles similar to Mot1p *in vivo*. *Mol Microbiol* **36**: 163-73.

Lemon, B. and R. Tjian. 2000. Orchestrated response: a symphony of transcription factors for gene control. *Genes Dev* **14**: 2551-69.

Leresche, A., V.J. Wolf, and J.M. Gottesfeld. 1996. Repression of RNA polymerase

II and III transcription during M phase of the cell cycle. *Exp Cell Res* **229**: 282-8.

Lin, Y.S., I. Ha, E. Maldonado, D. Reinberg, and M.R. Green. 1991. Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353**: 569-71.

Liu, Y., C. Kung, J. Fishburn, A.Z. Ansari, K.M. Shokat, and S. Hahn. 2004. Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. *Mol Cell Biol* **24**: 1721-35.

Long, J.J., A. Leresche, R.W. Kriwacki, and J.M. Gottesfeld. 1998. Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis. *Mol Cell Biol* **18**: 1467-76.

Love, J.D. and K.W. Minton. 1985. Screening of lambda library for differentially expressed genes using *in vitro* transcripts. *Anal Biochem* **150**: 429-41.

Lu, H., O. Flores, R. Weinmann, and D. Reinberg. 1991. The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc Natl Acad Sci U S A* **88**: 10004-8.

Lu, H., L. Zawel, L. Fisher, J.M. Egly, and D. Reinberg. 1992. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. *Nature* **358**: 641-5.

Luger, K. and T.J. Richmond. 1998. The histone tails of the nucleosome. *Curr Opin Genet Dev* **8**: 140-6.

Luscher, B. and R.N. Eisenman. 1992. Mitosis-specific phosphorylation of the nuclear oncoproteins Myc and Myb. *J Cell Biol* **118**: 775-84.

Luse, D.S. and G.A. Jacob. 1987. Abortive initiation by RNA polymerase II *in vitro* at the adenovirus 2 major late promoter. *J Biol Chem* **262**: 14990-7.

Ma, D., I. Olave, A. Merino, and D. Reinberg. 1996. Separation of the transcriptional coactivator and antirepression functions of transcription factor IIA. *Proc Natl Acad Sci U S A* **93**: 6583-8.

Macian, F., C. Lopez-Rodriguez, and A. Rao. 2001. Partners in transcription: NFAT and AP-1. *Oncogene* **20**: 2476-89.

Makkerh, J.P., C. Dingwall, and R.A. Laskey. 1996. Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Curr Biol* **6**: 1025-7.

Maldonado, E., I. Ha, P. Cortes, L. Weis, and D. Reinberg. 1990. Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex. *Mol Cell Biol* **10**: 6335-47.

Maldonado, E., M. Hampsey, and D. Reinberg. 1999. Repression: targeting the

heart of the matter. *Cell* **99**: 455-8.

Maldonado, E., R. Shiekhattar, M. Sheldon, H. Cho, R. Drapkin, P. Rickert, E. Lees, C.W. Anderson, S. Linn, and D. Reinberg. 1996. A human RNA polymerase II complex associated with SRB and DNA-repair proteins. *Nature* **381**: 86-9.

Malik, S., W. Gu, W. Wu, J. Qin, and R.G. Roeder. 2000. The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. *Mol Cell* **5**: 753-60.

Malik, S. and R.G. Roeder. 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. *Trends Biochem Sci* **25**: 277-83.

Marks, P.A., T. Miller, and V.M. Richon. 2003. Histone deacetylases. *Curr Opin Pharmacol* **3**: 344-51.

Marmorstein, R. and S.Y. Roth. 2001. Histone acetyltransferases: function, structure, and catalysis. *Curr Opin Genet Dev* **11**: 155-61.

Martinez, E., Q. Zhou, N.D. L'Etoile, T. Oelgeschlager, A.J. Berk, and R.G. Roeder. 1995. Core promoter-specific function of a mutant transcription factor TFIID defective in TATA-box binding. *Proc Natl Acad Sci U S A* **92**: 11864-8.

Martinez-Balbas, M.A., U.M. Bauer, S.J. Nielsen, A. Brehm, and T. Kouzarides. 2000. Regulation of E2F1 activity by acetylation. *Embo J* **19**: 662-71.

Martinez-Balbas, M.A., A. Dey, S.K. Rabindran, K. Ozato, and C. Wu. 1995. Displacement of sequence-specific transcription factors from mitotic chromatin. *Cell* **83**: 29-38.

Matsui, T., J. Segall, P.A. Weil, and R.G. Roeder. 1980. Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. *J Biol Chem* **255**: 11992-6.

McCracken, S. and J. Greenblatt. 1991. Related RNA polymerase-binding regions in human RAP30/74 and Escherichia coli sigma 70. *Science* **253**: 900-2.

Meinhart, A. and P. Cramer. 2004. Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors. *Nature* **430**: 223-6.

Meisterernst, M. and R.G. Roeder. 1991. Family of proteins that interact with TFIID and regulate promoter activity. *Cell* **67**: 557-67.

Meisterernst, M., A.L. Roy, H.M. Lieu, and R.G. Roeder. 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. *Cell* **66**: 981-93.

Meisterernst, M., G. Stelzer, and R.G. Roeder. 1997. Poly(ADP-ribose) polymerase enhances activator-dependent transcription *in vitro*. *Proc Natl Acad Sci U S A* **94**: 2261-5.

Mellon, I. and P.C. Hanawalt. 1989. Induction of the Escherichia coli lactose operon

selectively increases repair of its transcribed DNA strand. *Nature* **342**: 95-8.

Merino, A., K.R. Madden, W.S. Lane, J.J. Champoux, and D. Reinberg. 1993. DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* **365**: 227-32.

Mermelstein, F., K. Yeung, J. Cao, J.A. Inostroza, H. Erdjument-Bromage, K. Eagelson, D. Landsman, P. Levitt, P. Tempst, and D. Reinberg. 1996. Requirement of a corepressor for Dr1-mediated repression of transcription. *Genes Dev* **10**: 1033-48.

Michelotti, E.F., S. Sanford, and D. Levens. 1997. Marking of active genes on mitotic chromosomes. *Nature* **388**: 895-9.

Mizzen, C.A., X.J. Yang, T. Kokubo, J.E. Brownell, A.J. Bannister, T. Owen-Hughes, J. Workman, L. Wang, S.L. Berger, T. Kouzarides, Y. Nakatani, and C.D. Allis. 1996. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**: 1261-70.

Moore, M.S. 1998. Ran and nuclear transport. *J Biol Chem* **273**: 22857-60.

Moqtaderi, Z., Y. Bai, D. Poon, P.A. Weil, and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* **383**: 188-91.

Moreland, R.J., F. Tirode, Q. Yan, J.W. Conaway, J.M. Egly, and R.C. Conaway. 1999. A role for the TFIIH XPB DNA helicase in promoter escape by RNA polymerase II. *J Biol Chem* **274**: 22127-30.

Muchardt, C., J.C. Reyes, B. Bourachot, E. Leguoy, and M. Yaniv. 1996. The hbrm and BRG-1 proteins, components of the human SNF/SWI complex, are phosphorylated and excluded from the condensed chromosomes during mitosis. *Embo J* **15**: 3394-402.

Muldrow, T.A., A.M. Campbell, P.A. Weil, and D.T. Auble. 1999. MOT1 can activate basal transcription *in vitro* by regulating the distribution of TATA binding protein between promoter and nonpromoter sites. *Mol Cell Biol* **19**: 2835-45.

Muller, F. and L. Tora. 2004. The multicoloured world of promoter recognition complexes. *Embo J* **23**: 2-8.

Myers, L.C. and R.D. Kornberg. 2000. Mediator of transcriptional regulation. *Annu Rev Biochem* **69**: 729-49.

Naar, A.M., B.D. Lemon, and R. Tjian. 2001. Transcriptional coactivator complexes. *Annu Rev Biochem* **70**: 475-501.

Nakai, K. and M. Kanehisa. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14**: 897-911.

Narlikar, G.J., H.Y. Fan, and R.E. Kingston. 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**: 475-87.

Nelson, M. and P. Silver. 1989. Context affects nuclear protein localization in Saccharomyces cerevisiae. *Mol Cell Biol* **9**: 384-9.

Nickel, B.E., C.D. Allis, and J.R. Davie. 1989. Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. *Biochemistry* **28**: 958-63.

Nigg, E.A. 1993. Cellular substrates of p34(cdc2) and its companion cyclin-dependent kinases. *Trends Cell Biol* **3**: 296-301.

-. 2001. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* **2**: 21-32.

O'Brien, T., S. Hardin, A. Greenleaf, and J.T. Lis. 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. *Nature* **370**: 75-7.

Ogryzko, V.V., R.L. Schiltz, V. Russanova, B.H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**: 953-9.

Ohbayashi, T., M. Shimada, T. Nakadai, T. Wada, H. Handa, and T. Tamura. 2003. Vertebrate TBP-like protein (TLP/TRF2/TLF) stimulates TATA-less terminal deoxynucleotidyl transferase promoters in a transient reporter assay, and TFIIA-binding capacity of TLP is required for this function. *Nucleic Acids Res* **31**: 2127-33.

Ohkuma, Y. 1997. Multiple functions of general transcription factors TFIIE and TFIIH in transcription: possible points of regulation by trans-acting factors. *J Biochem (Tokyo)* **122**: 481-9.

Ohkuma, Y., S. Hashimoto, C.K. Wang, M. Horikoshi, and R.G. Roeder. 1995. Analysis of the role of TFIIE in basal transcription and TFIIH-mediated carboxy-terminal domain phosphorylation through structure-function studies of TFIIE-alpha. *Mol Cell Biol* **15**: 4856-66.

Ohno, M., M. Fornerod, and I.W. Mattaj. 1998. Nucleocytoplasmic transport: the last 200 nanometers. *Cell* **92**: 327-36.

Ornaghi, P., P. Ballario, A.M. Lena, A. Gonzalez, and P. Filetici. 1999. The bromodomain of Gcn5p interacts *in vitro* with specific residues in the N terminus of histone H4. *J Mol Biol* **287**: 1-7.

Orphanides, G., T. Lagrange, and D. Reinberg. 1996. The general transcription factors of RNA polymerase II. *Genes Dev* **10**: 2657-83.

Orphanides, G. and D. Reinberg. 2002. A unified theory of gene expression. *Cell* **108**: 439-51.

Ossipow, V., J.P. Tassan, E.A. Nigg, and U. Schibler. 1995. A mammalian RNA polymerase II holoenzyme containing all components required for promoter-specific transcription initiation. *Cell* **83**: 137-46.

Parsons, G.G. and C.A. Spencer. 1997. Mitotic repression of RNA polymerase II transcription is accompanied by release of transcription elongation complexes. *Mol Cell Biol* **17**: 5791-802.

Patturajan, M., R.J. Schulte, B.M. Sefton, R. Berezney, M. Vincent, O. Bensaude, S.L. Warren, and J.L. Corden. 1998. Growth-related changes in phosphorylation of yeast RNA polymerase II. *J Biol Chem* **273**: 4689-94.

Pederson, T. 1998. The plurifunctional nucleolus. *Nucleic Acids Res* **26**: 3871-6.

Pereira, L.A., M.P. Klejman, and H.T. Timmers. 2003. Roles for BTAF1 and Mot1p in dynamics of TATA-binding protein and regulation of RNA polymerase II transcription. *Gene* **315**: 1-13.

Peterson, C.L. 2002. HDAC's at work: everyone doing their part. *Mol Cell* **9**: 921-2.

Pham, A.D. and F. Sauer. 2000. Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in Drosophila. *Science* **289**: 2357-60.

Pirkkala, L., P. Nykanen, and L. Sistonen. 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *Faseb J* **15**: 1118-31.

Pollard, V.W., W.M. Michael, S. Nakielny, M.C. Siomi, F. Wang, and G. Dreyfuss. 1996. A novel receptor-mediated nuclear protein import pathway. *Cell* **86**: 985-94.

Poot, R.A., G. Dellaire, B.B. Hulsmann, M.A. Grimaldi, D.F. Corona, P.B. Becker, W.A. Bickmore, and P.D. Varga-Weisz. 2000. HuCHRAC, a human ISWI chromatin remodelling complex contains hACF1 and two novel histone-fold proteins. *Embo J* **19**: 3377-87.

Prelich, G. 1997. Saccharomyces cerevisiae BUR6 encodes a DRAP1/NC2alpha homolog that has both positive and negative roles in transcription *in vivo*. *Mol Cell Biol* **17**: 2057-65.

Prelich, G. and F. Winston. 1993. Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription *in vivo*. *Genetics* **135**: 665-76.

Prescott, D.M. and M.A. Bender. 1962. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exp Cell Res* **26**: 260-8.

Proudfoot, N.J., A. Furger, and M.J. Dye. 2002. Integrating mRNA processing with transcription. *Cell* **108**: 501-12.

Pugh, B.F. and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**: 1187-97.

Rabenstein, M.D., S. Zhou, J.T. Lis, and R. Tjian. 1999. TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family. *Proc Natl Acad Sci U S A* **96**: 4791-6.

Raff, J.W., R. Kellum, and B. Alberts. 1994. The Drosophila GAGA transcription factor is associated with specific regions of heterochromatin throughout the cell cycle. *Embo J* **13**: 5977-83.

Rani, P.G., J.A. Ranish, and S. Hahn. 2004. RNA polymerase II (Pol II)-TFIIF and Pol II-mediator complexes: the major stable Pol II complexes and their activity in transcription initiation and reinitiation. *Mol Cell Biol* **24**: 1709-20.

Ranish, J.A., N. Yudkovsky, and S. Hahn. 1999. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev* **13**: 49-63.

Rea, S., F. Eisenhaber, D. O'Carroll, B.D. Strahl, Z.W. Sun, M. Schmid, S. Opravil, K. Mechtler, C.P. Ponting, C.D. Allis, and T. Jenuwein. 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**: 593-9.

Reeder, R.H. and R.G. Roeder. 1972. Ribosomal RNA synthesis in isolated nuclei. *J Mol Biol* **67**: 433-41.

Reichelt, R., A. Holzenburg, E.L. Buhle, Jr., M. Jarnik, A. Engel, and U. Aebi. 1990. Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. *J Cell Biol* **110**: 883-94.

Reinberg, D., G. Orphanides, R. Ebright, S. Akoulitchev, J. Carcamo, H. Cho, P. Cortes, R. Drapkin, O. Flores, I. Ha, J.A. Inostroza, S. Kim, T.K. Kim, P. Kumar, T. Lagrange, G. LeRoy, H. Lu, D.M. Ma, E. Maldonado, A. Merino, F. Mermelstein, I. Olave, M. Sheldon, R. Shiekhattar, L. Zawel, and et al. 1998. The RNA polymerase II general transcription factors: past, present, and future. *Cold Spring Harb Symp Quant Biol* **63**: 83-103.

Roberts, B.L., W.D. Richardson, and A.E. Smith. 1987. The effect of protein context on nuclear location signal function. *Cell* **50**: 465-75.

Roeder, R.G. 1996. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* **21**: 327-35.

Rossignol, M., A. Keriel, A. Staub, and J.M. Egly. 1999. Kinase activity and phosphorylation of the largest subunit of TFIIF transcription factor. *J Biol Chem* **274**: 22387-92.

Rouleau, M., R.A. Aubin, and G.G. Poirier. 2004. Poly(ADP-ribosyl)ated chromatin

domains: access granted. *J Cell Sci* **117**: 815-25.

Rout, M.P. and G. Blobel. 1993. Isolation of the yeast nuclear pore complex. *J Cell Biol* **123**: 771-83.

Rowland, R.R. and D. Yoo. 2003. Nucleolar-cytoplasmic shuttling of PRRSV nucleocapsid protein: a simple case of molecular mimicry or the complex regulation by nuclear import, nucleolar localization and nuclear export signal sequences. *Virus Res* **95**: 23-33.

Roy, R., L. Schaeffer, S. Humbert, W. Vermeulen, G. Weeda, and J.M. Egly. 1994. The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. *J Biol Chem* **269**: 9826-32.

Sakurai, H., T. Ohishi, and T. Fukasawa. 1997. Promoter structure-dependent functioning of the general transcription factor IIE in Saccharomyces cerevisiae. *J Biol Chem* **272**: 15936-42.

Santos-Rosa, H., R. Schneider, A.J. Bannister, J. Sherriff, B.E. Bernstein, N.C. Emre, S.L. Schreiber, J. Mellor, and T. Kouzarides. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature* **419**: 407-11.

Sauer, F., J.D. Fondell, Y. Ohkuma, R.G. Roeder, and H. Jackle. 1995. Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta. *Nature* **375**: 162-4.

Schaeffer, L., R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J.H. Hoeijmakers, P. Chambon, and J.M. Egly. 1993. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. *Science* **260**: 58-63.

Scheer, U. and R. Hock. 1999. Structure and function of the nucleolus. *Curr Opin Cell Biol* **11**: 385-90.

Schlenstedt, G. 1996. Protein import into the nucleus. *FEBS Lett* **389**: 75-9.

Segil, N., M. Guermah, A. Hoffmann, R.G. Roeder, and N. Heintz. 1996. Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. *Genes Dev* **10**: 2389-400.

Segil, N., S.B. Roberts, and N. Heintz. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science* **254**: 1814-6.

Sekinger, E.A. and D.S. Gross. 2001. Silenced chromatin is permissive to activator binding and PIC recruitment. *Cell* **105**: 403-14.

Shermoen, A.W. and P.H. O'Farrell. 1991. Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. *Cell* **67**: 303-10.

Shiio, Y. and R.N. Eisenman. 2003. Histone sumoylation is associated with transcriptional repression. *Proc Natl Acad Sci U S A* **100**: 13225-30.

Shykind, B.M., J. Kim, and P.A. Sharp. 1995. Activation of the TFIID-TFIIA complex

with HMG-2. *Genes Dev* **9**: 1354-65.

Sidorova, J.M., G.E. Mikesell, and L.L. Breeden. 1995. Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. *Mol Biol Cell* **6**: 1641-58.

Singer, V.L., C.R. Wobbe, and K. Struhl. 1990. A wide variety of DNA sequences can functionally replace a yeast TATA element for transcriptional activation. *Genes Dev* **4**: 636-45.

Song, A., Q. Wang, M.G. Goebl, and M.A. Harrington. 1998. Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol Cell Biol* **18**: 4994-9.

Spencer, E., J. Jiang, and Z.J. Chen. 1999. Signal-induced ubiquitination of IkappaBalpha by the F-box protein Slimb/beta-TrCP. *Genes Dev* **13**: 284-94.

Sprang, S.R., K.R. Acharya, E.J. Goldsmith, D.I. Stuart, K. Varvill, R.J. Fletterick, N.B. Madsen, and L.N. Johnson. 1988. Structural changes in glycogen phosphorylase induced by phosphorylation. *Nature* **336**: 215-21.

Starr, D.B. and D.K. Hawley. 1991. TFIID binds in the minor groove of the TATA box. *Cell* **67**: 1231-40.

Steel, G.J., D.M. Fullerton, J.R. Tyson, and C.J. Stirling. 2004. Coordinated activation of Hsp70 chaperones. *Science* **303**: 98-101.

Stokes, D.G., K.D. Tartof, and R.P. Perry. 1996. CHD1 is concentrated in interbands and puffed regions of Drosophila polytene chromosomes. *Proc Natl Acad Sci U S A* **93**: 7137-42.

Strahl, B.D. and C.D. Allis. 2000. The language of covalent histone modifications. *Nature* **403**: 41-5.

Strahl, B.D., R. Ohba, R.G. Cook, and C.D. Allis. 1999. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. *Proc Natl Acad Sci U S A* **96**: 14967-72.

Struhl, K. 1984. Genetic properties and chromatin structure of the yeast gal regulatory element: an enhancer-like sequence. *Proc Natl Acad Sci U S A* **81**: 7865-9.

-. 1995. Yeast transcriptional regulatory mechanisms. *Annu Rev Genet* **29**: 651-74.

Stukenberg, P.T., K.D. Lustig, T.J. McGarry, R.W. King, J. Kuang, and M.W. Kirschner. 1997. Systematic identification of mitotic phosphoproteins. *Curr Biol* **7**: 338-48.

Svejstrup, J.Q., Z. Wang, W.J. Feaver, X. Wu, D.A. Bushnell, T.F. Donahue, E.C. Friedberg, and R.D. Kornberg. 1995. Different forms of TFIIH for transcription

and DNA repair: holo-TFIIH and a nucleotide excision repairosome. *Cell* **80**: 21-8.

Swedlow, J.R. and T. Hirano. 2003. The making of the mitotic chromosome: modern insights into classical questions. *Mol Cell* **11**: 557-69.

Terasima, T. and L.J. Tolmach. 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp Cell Res* **30**: 344-62.

Thebault, S., J. Basbous, B. Gay, C. Devaux, and J.M. Mesnard. 2000. Sequence requirement for the nucleolar localization of human I-mfa domain-containing protein (HIC p40). *Eur J Cell Biol* **79**: 834-8.

Thompson, C.M., A.J. Koleske, D.M. Chao, and R.A. Young. 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* **73**: 1361-75.

Timmers, H.T. and P.A. Sharp. 1991. The mammalian TFIID protein is present in two functionally distinct complexes. *Genes Dev* **5**: 1946-56.

Tong, J.K., C.A. Hassig, G.R. Schnitzler, R.E. Kingston, and S.L. Schreiber. 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature* **395**: 917-21.

Tora, L. 2002. A unified nomenclature for TATA box binding protein (TBP)-associated factors (TAFs) involved in RNA polymerase II transcription. *Genes Dev* **16**: 673-5.

Tsukiyama, T. 2002. The *in vivo* functions of ATP-dependent chromatin-remodelling factors. *Nat Rev Mol Cell Biol* **3**: 422-9.

Tupler, R., G. Perini, and M.R. Green. 2001. Expressing the human genome. *Nature* **409**: 832-3.

Van Dyke, M.W., R.G. Roeder, and M. Sawadogo. 1988. Physical analysis of transcription preinitiation complex assembly on a class II gene promoter. *Science* **241**: 1335-8.

Vandel, L. and T. Kouzarides. 1999. Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase. *Embo J* **18**: 4280-91.

Veenstra, G.J. and A.P. Wolffe. 2001. Gene-selective developmental roles of general transcription factors. *Trends Biochem Sci* **26**: 665-71.

Verrijzer, C.P., K. Yokomori, J.L. Chen, and R. Tjian. 1994. Drosophila TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. *Science* **264**: 933-41.

Visintin, R., E.S. Hwang, and A. Amon. 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* **398**: 818-23.

Walker, S.S., J.C. Reese, L.M. Apone, and M.R. Green. 1996. Transcription

activation in cells lacking TAFIIS. *Nature* **383**: 185-8.

Walker, S.S., W.C. Shen, J.C. Reese, L.M. Apone, and M.R. Green. 1997. Yeast TAF(II)145 required for transcription of G1/S cyclin genes and regulated by the cellular growth state. *Cell* **90**: 607-14.

Wang, W., M. Carey, and J.D. Gralla. 1992a. Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. *Science* **255**: 450-3.

Wang, W., J.D. Gralla, and M. Carey. 1992b. The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. *Genes Dev* **6**: 1716-27.

Watrin, E. and V. Legagneux. 2003. Introduction to chromosome dynamics in mitosis. *Biol Cell* **95**: 507-13.

Weil, P.A., D.S. Luse, J. Segall, and R.G. Roeder. 1979. Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA. *Cell* **18**: 469-84.

Wells, J., P. Held, S. Illenye, and N.H. Heintz. 1996. Protein-DNA interactions at the major and minor promoters of the divergently transcribed dhfr and rep3 genes during the Chinese hamster ovary cell cycle. *Mol Cell Biol* **16**: 634-47.

Wen, W., J.L. Meinkoth, R.Y. Tsien, and S.S. Taylor. 1995. Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**: 463-73.

White, R.J., T.M. Gottlieb, C.S. Downes, and S.P. Jackson. 1995. Cell cycle regulation of RNA polymerase III transcription. *Mol Cell Biol* **15**: 6653-62.

White, R.J., B.C. Khoo, J.A. Inostroza, D. Reinberg, and S.P. Jackson. 1994. Differential regulation of RNA polymerases I, II, and III by the TBP-binding repressor Dr1. *Science* **266**: 448-50.

Whitmarsh, A.J. and R.J. Davis. 2000. Regulation of transcription factor function by phosphorylation. *Cell Mol Life Sci* **57**: 1172-83.

Willy, P.J., R. Kobayashi, and J.T. Kadonaga. 2000. A basal transcription factor that activates or represses transcription. *Science* **290**: 982-5.

Wobbe, C.R. and K. Struhl. 1990. Yeast and human TATA-binding proteins have nearly identical DNA sequence requirements for transcription *in vitro*. *Mol Cell Biol* **10**: 3859-67.

Workman, J.L. and R.E. Kingston. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* **67**: 545-79.

Xie, J., M. Collart, M. Lemaire, G. Stelzer, and M. Meisterernst. 2000. A single point mutation in TFIIA suppresses NC2 requirement *in vivo*. *Embo J* **19**: 672-82.

Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B.M. Emerson, M. Montminy, and R.M. Evans. 2001. A transcriptional switch mediated by cofactor methylation. *Science* **294**: 2507-11.

Xue, Y., J. Wong, G.T. Moreno, M.K. Young, J. Cote, and W. Wang. 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* **2**: 851-61.

Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A.M. Manning, J.S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah. 1998. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature* **396**: 590-4.

Yokomori, K., A. Admon, J.A. Goodrich, J.L. Chen, and R. Tjian. 1993. Drosophila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev* **7**: 2235-45.

Yudkovsky, N., J.A. Ranish, and S. Hahn. 2000. A transcription reinitiation intermediate that is stabilized by activator. *Nature* **408**: 225-9.

Zawel, L. and D. Reinberg. 1995. Common themes in assembly and function of eukaryotic transcription complexes. *Annu Rev Biochem* **64**: 533-61.

Zhang, J. and J.L. Corden. 1991. Identification of phosphorylation sites in the repetitive carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. *J Biol Chem* **266**: 2290-6.

Zhu, A. and M.A. Kuziora. 1996. Homeodomain interaction with the beta subunit of the general transcription factor TFIIE. *J Biol Chem* **271**: 20993-6.

Zhu, J. and F. McKeon. 1999. NF-AT activation requires suppression of Crm1-dependent export by calcineurin. *Nature* **398**: 256-60.

Zhu, J., F. Shibasaki, R. Price, J.C. Guillemot, T. Yano, V. Dotsch, G. Wagner, P. Ferrara, and F. McKeon. 1998. Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. *Cell* **93**: 851-61.

PUBLICATIONS

The work presented in this thesis contributed in the following manuscripts:

Gilfillan, S., G. Stelzer, E. Piaia, M.G. Hofmann and M. Meisterernst. 2005. Efficient binding of NC2-TBP to DNA in the absence of TATA. *J Biol Chem* **280**: 6222-30

Previous work has been published in:

Loregian, A., E. Piaia, E. Cancellotti, E. Papini, H.S. Marsden, and G. Palu. 2000. The catalytic subunit of herpes simplex virus type 1 DNA polymerase contains a nuclear localization signal in the UL42-binding region. *Virology* **273**: 139-148.

ACKNOWLEDGMENTS

I would like to thank my supervisor Michael Meisterernst for giving me the opportunity to work in his laboratory under his guidance.
Prof. Peter Becker kindly provided me financial support in the last period of my work.
Walter Murani and Juergen Hass introduced me to the RGB world of confocal microscopy.
A BIG thank to all the people in the lab, that through the years provided the conditions for an almost organized lab work and a cooperative atmosphere: Gerhard and Barbara, who introduced me to the biochemistry world, Gertraud, the latin corner, Lucia, Lisa, Gema, spring of good spirits; Stefan, for providing good music for pipetting, Michi, for interesting scientific discussions, Tom-Thomas-Tomaso, Eric, Gabi, Andreas and Andrea.
I have a huge debt of gratitude to the best bench-mate I could ever find. Siv, your sincere support and positive thinking was always motivating me for never giving up.
I especially thank Lisa, whose good will I always admired, for being always helpful with suggestions in lab and non-lab issues.
I am indebted with Laura for hosting me in her lab while I was writing this thesis and to Ruco and Pleuni for interesting discussions.
Grazie Lino for always taking care of me and being so cheerful. Your computer knowledge contributed enormously to solve many problems.

Finally, many thanks to my two families, Gregorio, Giuliana, Tomaso, Federica, nonna, Marisa, Giovanni, Marta, Piero, Cecilia for their support and never ending storage of good wine, sopresse, cheese et al., that contributed significantly to the survival.

CURRICULUM VITAE

Personal Data

First name:	Elisa
Surname:	Piaia
Date of birth:	13-03-1974
Place of birth:	Montebelluna (TV), Italy.
Nationality:	Italian
Marital status:	married
Address:	Daiser str., 16 81371 Muenchen Germany
Tel. at home:	+49-(0)89-76754900
Tel. at work:	+49-(0)89-7099543
e-mail:	e.piaia@web.de
Home address:	via S. Caterina da Siena, 59 31044 Montebelluna (Treviso) Italy

Education

1988 – 1993:	High School Graduation Liceo classico "Manara Valgimigli", Montebelluna (TV), Italy
1993 – 1999:	First degree in Biological Sciences (score 110/110) University of Padua, Italy
Since 01.05.2000:	PhD student in Michael Meisterernst group, at the Genzenrtum, LMU-Ludwig Maximilians Universitaet Munich and from June 2001, at the GSF-Forschungszentrum fuer Umwelt und Gesundheit, Munich