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Functional Antagonism of the RNA Polymerase II Holoenzyme by Negative Regulators

by

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B.A., Biology
College of St. Catherine, 1990

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 1997

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To my family for their love and support.

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by

Ellen L. Gadbois

Submitted to the Department of Biology on July 22, 1997 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology at the Massachusetts Institute of Technology

Abstract

The regulation of class II gene expression requires a complex interplay of stimulatory and inhibitory factors. Transcription initiation in yeast involves the recruitment of the RNA polymerase II holoenzyme, which contains RNA polymerase II, general transcription factors, and SRB proteins. Genetic suppression analysis of the yeast *SRB4* gene, which plays a positive and essential role in transcription, led to the identification of several general negative regulators. Mutations in either subunit of the yeast homologue of the human negative regulator NC2 suppress mutations in *SRB4*. Global defects in mRNA synthesis caused by the defective yeast holoenzyme are alleviated by an NC2 suppressing mutation *in vivo*, indicating that yeast NC2 is a global negative regulator of class II transcription. Mutations in the negative regulators *NOT1* and *NOT3* also alleviate the *srb4* defect. These results imply that relief from repression at class II promoters is a general feature of gene activation *in vivo*. To begin extending this work into mammalian cells, a mammalian SRB7 homologue was identified and used as a marker in the purification of a mammalian RNA polymerase II holoenzyme.

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Table of Contents

Title Page	1
Dedication	2
Abstract	3
Table of Contents	4
Chapter 1:	
Introduction: Transcription initiation and general negative regulators.....	5
Chapter 2:	
Functional antagonism between RNA polymerase II holoenzyme and global negative regulator NC2 <i>in vivo</i>	51
Chapter 3:	
Functional antagonism between RNA polymerase II holoenzyme and Not proteins <i>in vivo</i>	80
Chapter 4:	
Discussion: General negative regulation.....	97
Chapter 5:	
A mammalian SRB protein associated with an RNA polymerase II holoenzyme.....	105
Appendix A:	
Extragenic suppressors of an <i>srb4</i> conditional mutation.....	127

Chapter 1

Introduction: Transcription Initiation and General Negative Regulators

Overview

In this chapter, I review the factors involved in transcription initiation, and focus on general negative regulators in the yeast *S. cerevisiae*. I also explain my specific contributions to the projects described in this thesis.

Regulation of transcription

The phenotype of an organism is determined largely by its pattern of gene expression. Gene expression is regulated by elements within promoters that are bound by proteins which can activate or repress transcription [reviewed in (1-3)]. Activators are composed of two domains: a DNA-binding domain and an activation domain [reviewed in (4)]. The DNA-binding region binds to specific sequences within promoters called upstream activating sequences (UASs) in yeast and enhancers in higher eukaryotes. The activation domain contacts components of the general transcription apparatus. These interactions are postulated to result in the recruitment of the transcription machinery to promoters, the stabilization of the initiation apparatus, and the stimulation of post-initiation events. Repression of transcription is not as well understood, due in part to more interest in activation and difficulties in studying negative regulation.

Early studies of gene expression in the *lac* operon demonstrate the importance of negative regulation in prokaryotes (5). Recent studies in yeast reveal that individual gene expression varies from 0.3 to 200 transcripts per cell, with 20% of protein-encoding genes not expressed at detectable levels under standard laboratory conditions (6, 7). In higher eukaryotes, an even larger percentage of the genome is repressed (8). In order to understand how this widespread gene repression is achieved, we must consider the functions

of the general transcription factors and how they are influenced by the general negative regulators of the cell.

Transcription initiation

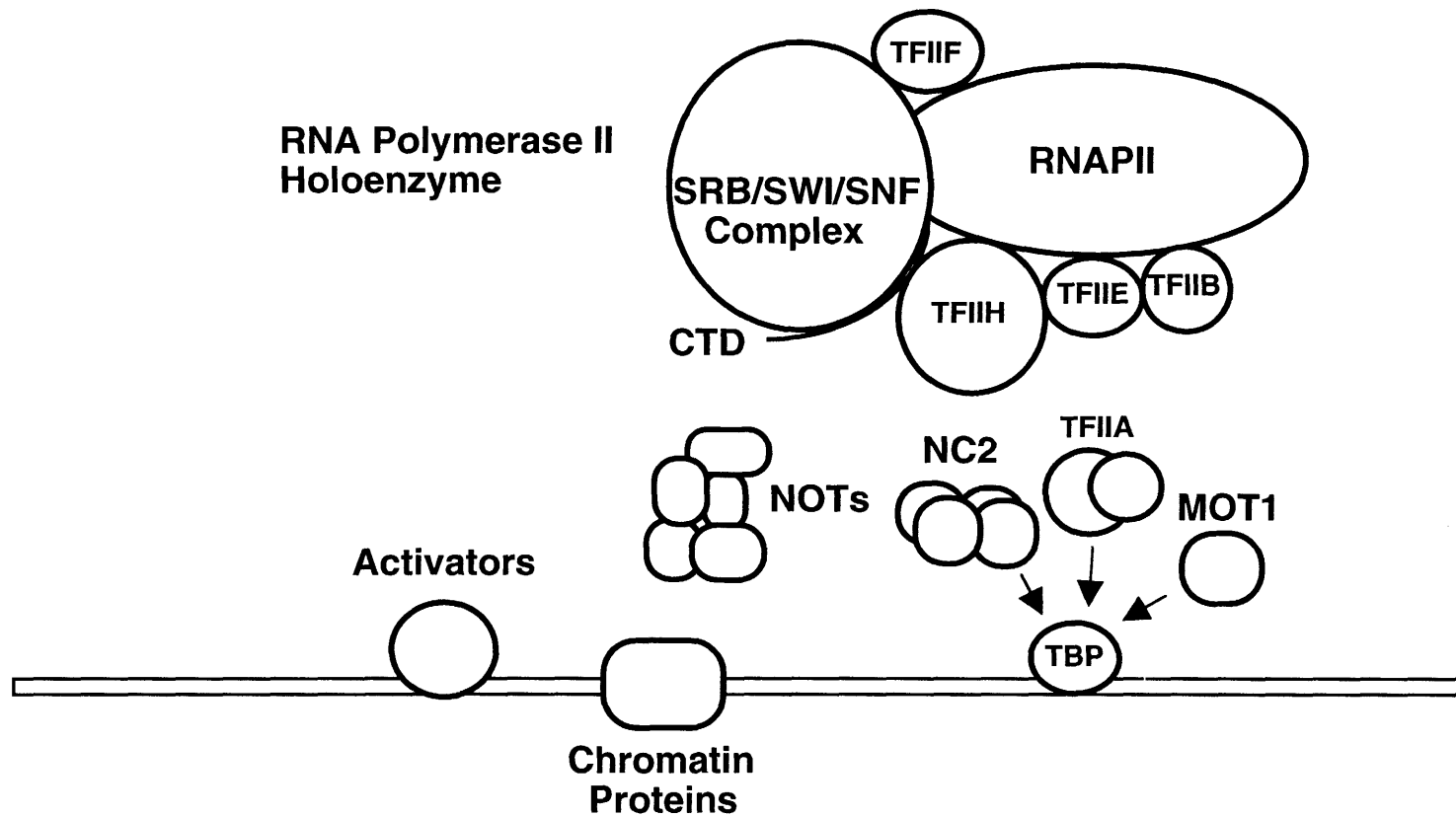
A central component of the general transcription apparatus is a large multisubunit complex called the RNA polymerase II holoenzyme. The *S. cerevisiae* holoenzyme contains RNA polymerase II; the general transcription factors TFIIB, TFIIE, TFIIIF, and TFIIH; and a subcomplex containing SRBs, Swi/Snf proteins, and other regulatory factors (Fig. 1). The holoenzyme binds to the TATA-binding protein TBP at promoter elements and initiates mRNA synthesis. Described below are the components of the general transcription initiation apparatus and characteristics of the RNA polymerase II holoenzyme.

RNA polymerase II. Three different RNA polymerases catalyze gene expression in eukaryotes. RNA polymerase I transcribes the class I genes encoding large ribosomal RNA, RNA polymerase II transcribes the class II protein-encoding genes, and RNA polymerase III transcribes the class III genes encoding tRNA and the 5S ribosomal RNA (9).

In yeast, core RNA polymerase II is a 500 kDa complex containing 12 protein subunits [reviewed in (10)]. All but two of the genes encoding these subunits are essential for cell viability (10-13). Two of the subunits, Rpb4 and Rpb7, appear to form a subcomplex that can dissociate from the other subunits (14). The relative amounts of Rpb4 and Rpb7 increase during physiological stress conditions (15).

The subunits of RNA polymerases show strong evolutionary conservation. Five of the yeast RNA polymerase II subunits are also found in RNA polymerases I and III (16, 17). The 2 largest subunits of yeast RNA

Fig. 1 Regulation of Transcription Initiation *in vivo*



polymerase II, Rpb1 and Rpb2, have significant homology to the 2 largest subunits of RNA polymerase II in other eukaryotes and to the *E.coli* RNA polymerase core subunits β' and β (10). The core *E.coli* RNA polymerase enzyme requires a σ subunit for selective promoter recognition (18). RNA polymerase II also requires additional components, the general transcription factors, for selective transcription initiation *in vitro*.

The carboxy-terminal repeat domain (CTD) of the largest subunit of eukaryotic RNA polymerase II is an intriguing and conserved domain [reviewed in (19, 20)]. The CTD contains the consensus heptapeptide sequence YSPTSPS, which is repeated 26 to 52 times depending on the organism (21-23). The number of repeats within the CTD increases with the genomic complexity of the organism. The CTD is essential for cell viability in several organisms (24-26). Partial truncations of the CTD cause general defects in cell growth (24) and transcription initiation (27). Similar mutations reduce the response to transcriptional activators at a subset of genes in yeast (28, 29) and mammalian cells (30). The requirement for the CTD in *in vitro* transcription assays varies by promoter (27, 31-33). Thus, the CTD appears to be involved in transcription and the response to activators at a subset of promoters.

A population of the RNA polymerase II in yeast and mammalian cells is highly phosphorylated at the CTD (34, 35). Phosphorylation has been reported to occur at the second and fifth serine residues (36), and also at the tyrosine residues (37). Phosphorylation causes the CTD to adopt an extended structure *in vitro* (38). Correlative evidence suggests that CTD phosphorylation is involved in promoter clearance. RNA polymerase II containing an unphosphorylated CTD is preferentially recruited to promoters (39, 40). The form of polymerase that has initiated transcription or is engaged in elongation contains a phosphorylated CTD (34, 41). However,

phosphorylation of the CTD is not required for transcription in one highly purified system (42). This may be due to the absence of negative factors which are normally overcome by CTD phosphorylation. Indeed, a crude yeast transcription system is sensitive to a kinase inhibitor while a highly purified system is not (43).

Several different kinases directly phosphorylate the CTD *in vitro* or affect CTD phosphorylation *in vivo*. The RNA polymerase II holoenzyme contains two CTD kinases: the general transcription factor TFIIF and the CTD-associated SRB10 protein. TFIIF phosphorylates the CTD *in vitro* (44, 45), but the physiological relevance of the reaction is not known. RNA polymerase II holoenzyme containing a mutant form of SRB10 has a 10-fold lower kinase activity *in vitro* (46), but no effect on CTD phosphorylation *in vivo* has yet been demonstrated. The yeast Ctk1 kinase phosphorylates the CTD *in vitro* (47), and a *CTK1* deletion shows a slight decrease in the levels of phosphorylated CTD *in vivo* (48). A mammalian Cdc2 (49), and *Drosophila* elongation factor (50) also phosphorylate the CTD *in vitro*. Although it is not clear how the CTD becomes phosphorylated *in vivo*, this data suggests that there may be several kinases involved.

The general transcription factors.

Early reconstitution experiments of transcription with RNA polymerase II identified a group of general transcription factors required for selective transcription initiation *in vitro*. These factors include TFIIA, TFIIB, TFIID (containing TBP), TFIIE, TFIIIF, and TFIIF [reviewed in (51)]. Most of the general transcription factors show high amino acid sequence homology between different eukaryotic organisms. *In vitro* studies demonstrate that the factors can assemble sequentially onto promoter elements [reviewed in (52, 53)]. In these experiments, TFIID or TBP binds to the TATA element of the

promoter, then TFIIA and TFIIB each bind to TBP-DNA, an RNA polymerase II-TFIIF complex is next recruited (partly through TFIIB contacts), and finally TFIIE and TFIIH join the preinitiation complex. While this sequential assembly may occur at some promoters *in vivo*, general factors can be found associated with RNA polymerase II in the absence of DNA as an RNA polymerase II holoenzyme.

TBP. The initiating event in class II transcription is the binding of TBP to a weakly conserved promoter element called the TATA box (54). TBP itself, like RNA polymerase II, is highly conserved throughout evolution (55). Human and yeast TBP are interchangeable in *in vitro* transcription reactions (56, 57), and a human TBP derivative functions in yeast cells (58). TBP is also a component of the RNA polymerase I factor SL1 (59) and the RNA polymerase III factor TFIIB (60).

Crystal structures of TBP provide insight into TBP function. The TBP protein has a symmetrical saddle shape (61, 62). TBP contacts the minor groove of the TATA element and severely bends the DNA (63, 64). Co-crystal structures reveal that TFIIA (65) and TFIIB (66) bind to opposite surfaces on TBP and have fairly small interaction surfaces. Whereas TBP is relatively small (27 kDa in yeast, 37 kDa in humans), it contacts a large number of transcription factors. Substoichiometric amounts of TBP are found in highly purified yeast RNA polymerase II holoenzyme (67), suggesting that TBP loosely associates with the holoenzyme.

TFIID. TBP can be purified in association with a set of proteins called TBP-associated factors (TAF_{II}s) in a complex called TFIID [reviewed in (68, 69)]. TFIID complexes have been isolated from yeast (70, 71), *Drosophila* (72), and human cells (73-75). In several *Drosophila* (72, 76, 77) and human (78) *in vitro* transcription systems, the response to activators is lost if TBP is

substituted for TFIID. A large number of activators directly interact with components of TFIID *in vitro* (79-83). These *in vitro* results suggest that TFIID plays an important role in activation of transcription.

In vivo analysis of TAF_{II}s reveals a different picture of TFIID function. Conditional TAF_{II} mutations and depletion of TAF_{II} proteins in yeast do not show global defects in class II transcription activation (84-86). Instead, TAF_{II} depletion affects the basal transcription of a small number of promoters. Interestingly, particular conditional TAF_{II} mutations show cell cycle arrest phenotypes (84, 86, 87). Consistent with this observation, a conditional mutation in mammalian TAF_{II}250 affects the transcription of several cyclin genes (88, 89). These results suggest that TAF_{II}s play a role in transcription at a limited number of genes that include cell-cycle regulators.

Some of the promoters affected by TAF_{II}s lack classical TATA elements. Mammalian promoters with no apparent TATA elements direct transcription with start site elements called initiators (90). Several initiator-directed *in vitro* transcription systems require TFIID and cannot utilize TBP (78, 91, 92). Similarly, a *Drosophila* gene with multiple promoter elements requires TFIID for selective promoter utilization (93). *In vivo* transcription of several yeast genes containing non-consensus TATA elements decreases when TAF_{II}s are depleted (85). TAF_{II}s may have repressive functions as well as stimulatory ones. *Drosophila* TAF_{II}230 inhibits the binding of TBP to DNA (94) and competes with the viral activator VP16 for TBP-binding (95).

TBP can also be purified in several other complexes that are involved in class II transcription. The TBP-Mot1 and B-TFIID complexes are discussed later in the context of negative regulation. Other TBP-interacting proteins include Spt3 and topoisomerase I. Mutations in *SPT3* suppress Ty transposable element insertion at several class II promoters (96), indicating

the involvement of Spt3 in transcription initiation. *spt3* alleles also suppress a mutation in the gene encoding TBP (97). Consistent with that result, Spt3 and TBP can be co-immunoprecipitated (97). Still, it is not clear how Spt3 affects TBP function. Topoisomerase I also interacts with TBP and represses basal transcription while stimulating activated transcription (98, 99). It is not known whether this activity occurs *in vivo*, and no genetic data links topoisomerase I to TBP.

TFIIB. TFIIB is composed of a single subunit (52). In yeast, the 38 kD TFIIB protein (100) is encoded by an essential gene (101). TFIIB binds to TBP-DNA complexes (102) and to RNA polymerase II in the absence of DNA (100, 103). TFIIB and RNA polymerase II are both involved in transcriptional start site selection, as shown by analysis of transcription start sites in mutants (101, 104, 105) and experiments combining transcription factors from different species *in vitro* (106).

The activator protein VP16 binds directly to TFIIB *in vitro* (107). Transcriptional activation by VP16 is dependent on its interaction with TFIIB (108), which results in increased recruitment of pre-initiation complexes (109) and a conformational change in TFIIB (110). Thus, TFIIB is involved in multiple steps of transcription initiation.

TFIIF. Mammalian TFIIF is composed of two subunits (111). Yeast TFIIF contains homologues of the mammalian subunits, and the genes encoding them are essential for viability (112, 113). Yeast TFIIF also contains an additional weakly associated subunit that is encoded by a nonessential gene (112), and is a shared subunit with the Swi/Snf complex (114) and TFIID (112).

TFIIF binds tightly to RNA polymerase II and reduces the affinity of polymerase for free DNA (115). It has limited homology to the bacterial σ factors (116) which also function in promoter selection (18). TFIIF can confer

responsiveness to a transcriptional activator *in vitro* (117). Besides its function in initiation, TFIIF stimulates elongation by RNA polymerase II *in vitro* (118, 119).

TFIIH. TFIIH is one of the more complex general transcription factors. It contains DNA repair, DNA helicase, and CTD kinase activities [reviewed in (120)]. Yeast TFIIH includes a subcomplex containing proteins involved in nucleotide excision repair and the TFIK subcomplex, which contains a kinase/cyclin pair (121-123). Different forms of TFIIH may be utilized for different functions: the TFIK subcomplex is required for transcription *in vitro*, while a form of TFIIH lacking TFIK is active in DNA repair (121).

The TFIK component of yeast TFIIH contains the kinase Kin28 (123) and the cyclin Ccl1 (124). Similarly, mammalian TFIIH contains the kinase-cyclin pair Cdk7 (MO15)/cyclin H, which has Cdk-activating kinase (CAK) activity *in vitro* (125-127). This activity suggests a link between the cell cycle and transcription. However, CAK activity in yeast is found in a complex distinct from yeast Kin28/Ccl1 (128-130). Neither biochemical nor genetic experiments support a role for yeast Kin28/Ccl1 involvement in the cell cycle (131).

There are several *in vitro* substrates for the TFIIH kinase activity, but it is not clear if any are physiologically relevant. Mammalian (45) and yeast (44) TFIIH, as well as the yeast TFIK subcomplex (123), can phosphorylate the CTD *in vitro*. TFIIH can phosphorylate other components of the transcription apparatus as well (132). Whatever the target, the kinase activity of TFIIH is required for transcription in crude *in vitro* systems (133), possibly to overcome negative regulation.

Numerous activators that stimulate transcriptional elongation bind to TFIIH (134, 135). The HIV activator Tat stimulates CTD phosphorylation by

TFIIH and elongation by the transcriptional apparatus *in vitro* (136). This correlation provides additional support for the involvement of CTD phosphorylation in promoter clearance.

TFIIE. This factor is a two-subunit complex in both yeast (137) and mammalian cells (138). Some of the known functions of TFIIE involve the regulation of TFIIH. TFIIE interacts with TFIIH (103), stimulates the CTD-kinase activity of TFIIH (45, 139), and inhibits TFIIH helicase activity (140). TFIIE appears to have independent functions; TFIIE, but not TFIIH, is required for *in vitro* transcription of a viral promoter (141). TFIIE is also important for transcriptional inhibition by the *Drosophila* Kruppel repressor (142).

TFIIA. Yeast TFIIA is composed of 14 and 32 kDa subunits (143) encoded by essential genes (144). Human TFIIA contains three subunits (145), with the larger two derived from a single precursor (146-148). TFIIA binds to TBP (143, 149) and stabilizes the TBP-DNA interaction (145, 150) TFIIA can overcome repression *in vitro* by a variety of TBP-binding negative regulators (99, 151-153). Like other general factors, TFIIA enhances the activity of many activator proteins (154-157). Fusing an activation-deficient TBP mutant to TFIIA restores activation *in vivo* (158). Mutations in TFIIA are synthetically lethal with the TBP-binding protein Spt3 (159).

There is a variable requirement for TFIIA among different *in vitro* transcription systems. Highly purified yeast (160) and mammalian (161) systems do not require TFIIA whereas nuclear extracts depleted for TFIIA show significantly reduced transcription (146, 155, 162). One system is stimulated by TFIIA in the presence of TFIID, but not TBP (145). The systems which require TFIIA may include negative regulators that are overcome by TFIIA function.

SRB proteins. The SRBs are a group of regulatory proteins which are involved in CTD function. The *SRB* (suppressors of RNA polymerase B or II) genes were cloned as allele-specific suppressors of the cold-sensitive phenotype of a CTD truncation mutation (46, 163-165). This genetic link is supported by biochemistry; the SRBs bind to a CTD affinity column (164) and are dissociated from RNA polymerase II by antibodies against the CTD (165, 166).

Individual SRBs can be described as either positive or negative regulators of transcription. The CTD-suppressing alleles of *SRB2, 4, 5,* and *6* include dominant, gain-of-function alleles (164), while *srb8, 9, 10,* and *11* suppressing alleles are all recessive, loss-of-function mutations (165). *SRB10* and *11* encode a kinase-cyclin pair (46).

Mutations in *SRB2, 4, 5,* or *6* cause severe transcriptional defects. *SRB2* and *SRB5* are required for preinitiation complex formation and efficient basal and activated transcription in crude nuclear extracts (164). Conditional mutations in the essential *SRB4* and *SRB6* genes result in rapid and global decreases of class II mRNA synthesis (167). These similarities are reflected in protein interactions: *SRB2, 4, 5,* and *6* form a complex *in vitro* (S. Koh, unpublished data). *SRB2* and *5* interact with each other, as do *SRB4* and *6*. These pairs may be brought together by the interaction of *SRB2* and *4*. *In vivo*, *SRB2* protein levels are greatly reduced in an *SRB5* deletion strain (164).

Strains containing deletions of *SRB8, 9, 10,* or *11* all exhibit similar slow-growth and flocculence phenotypes. Mutations in any of these SRBs suppress a mutation in *Snf1* (168, 169), a kinase involved in release from glucose repression. Mutations in *SRB8* and *SRB10* suppress a mutation in the gene encoding the $\alpha 2$ repressor of MATa-specific genes (170). *SRB10* is also required for meiotic mRNA stability in glucose-containing media (171). It is

not clear whether these effects are due to direct repression of transcription by SRBs. Although SRB10 is an attractive candidate for a CTD kinase and an *srb10* mutant allele reduces holoenzyme CTD kinase activity, *srb10* mutant alleles do not show defects in basal or activated transcription *in vitro* (46).

RNA polymerase II holoenzyme. Attempts to purify a complex of SRB proteins led to the purification of a >1 mDa complex called the RNA polymerase II holoenzyme (67). The most complete form of yeast holoenzyme contains RNA polymerase II; TFIIB, TFIIH; and a separable subcomplex containing all of the SRBs, Swi/Snf proteins, TFIIF, and other regulatory proteins [reviewed in (172, 173)]. TFIIE co-immunoprecipitates with the yeast holoenzyme in early fractions of the purification, suggesting that TFIIE is also a component (C. Wilson, unpublished data). The content of general transcription factors varies among different purifications of yeast holoenzyme (166, 174). This may be due to the existence of multiple forms of holoenzyme and differences in purification techniques. All reported forms of holoenzymes contain an SRB protein and RNA polymerase II.

SRBs are a hallmark of the holoenzyme, as the majority of the cellular SRB protein is present in a yeast holoenzyme preparation (67). In contrast, only 20% of RNA polymerase II is estimated to exist in an SRB-containing holoenzyme (67). However, the holoenzyme appears to be the functional form of yeast RNA polymerase II for class II transcription initiation, since *srb4* and *srb6* conditional mutant alleles show general decreases in mRNA transcription (167). Perhaps the other 80% of RNA polymerase II exists in elongating complexes lacking SRBs.

The RNA polymerase II holoenzyme is responsive to the acidic activator Gal4-VP16 in yeast *in vitro* transcription systems (67, 166). A reconstituted system containing general transcription factors and RNA

polymerase II does not respond to activators unless supplemented with an SRB-containing holoenzyme subcomplex (166). This SRB subcomplex interacts directly with Gal4-VP16 *in vitro* (165), as does the SRB4 protein (S. Koh, unpublished data).

A number of other regulatory proteins are present in the SRB-containing subcomplex of the holoenzyme. These include the 11 Swi/Snf chromatin remodeling proteins (175). Both the RNA polymerase II holoenzyme and a separately purified Swi/Snf complex exhibit ATP-dependent nucleosome disruption activities (175-178). *SWI/SNF* genes are required for the normal expression of a number of genes *in vivo* [reviewed in (179)].

Other SRB subcomplex components include Gal11 (166), Sin4 and Rgr1 (180), and Rox3 (181). Gal11 is required for the proper expression of a broad spectrum of genes *in vivo* (182), and a mutation in *GAL11* suppresses a mutation in the *GAL4* activator (183). Sin4 has both positive and negative effects on a number of genes *in vivo* (169, 170, 184-188), which could result from its proposed role as a modifier of chromatin structure (189). *RGR1* interacts genetically with the *SIN4* gene and *rgr1* and *sin4* mutants show similar defects (186, 188). Rox3 also has differential effects and appears to play a role in stress responses (169, 190-192). In summary, the yeast RNA polymerase II holoenzyme is composed of a diverse group of regulators which allow for sophisticated regulation of a complex genome.

Mammalian RNA polymerase II holoenzymes have recently been purified, but are less well characterized than the yeast versions (193-196). An SRB7 mammalian homologue has been identified (194) and is present in all of the mammalian holoenzymes (193-196). One of the holoenzymes was purified by immunoprecipitation directly from a nuclear extract and contains

all of the general transcription factors, including TFIID (193). This suggests that other holoenzyme purifications have partially disrupted an even larger complex of general transcription factors.

General negative regulators

A variety of negative regulators or effectors of class II gene expression exist in eukaryotic cells [reviewed in (197)]. Several of these factors influence the expression of a large number of genes so they are often referred to as global or general negative regulators. Among the most studied of these factors in yeast are NC2, Mot1, Nots, and chromatin components (Fig. 1). NC2, Mot1, and the Nots all have physical or genetic interactions with TBP. These factors are introduced here and are further discussed in Chapter 4. Since these factors are not fully understood, the use of the term “regulator” here does not necessarily imply that the primary role of these proteins is to regulate transcription, but could instead be the indirect effect of a different function, such as chromatin modulation.

NC2. NC2 (Dr1·DRAP1) is a negative regulator of transcription that binds TBP on promoter DNA and represses transcription *in vitro* [reviewed in (3)]. The factor was originally purified from mammalian cell extracts by two independent groups. A group led by Robert Roeder named the factor NC2 and identified two subunits, NC2 α and NC2 β , of 20 and 31 kDa (198). Another group led by Danny Reinberg purified a factor they named Dr1 (199), which is identical to NC2 β . The Reinberg group later found an associated protein, DRAP1 (200), which is identical to NC2 α . For the purposes of this review, I will refer to the complex as NC2. Both NC2 subunits are required for maximal binding of NC2 to TBP and repression of transcription *in vitro* (200-202). A yeast form of NC2 was recently identified (203-206) and is a general negative

regulator of class II transcription (204). The human NC2 genes can replace the yeast genes *in vivo* (205), demonstrating the high conservation of both the general transcription apparatus and NC2.

NC2 represses basal, and to varying degrees, activated transcription *in vitro* at a wide variety of mammalian, viral, and yeast promoters (151, 198, 200, 201, 203, 204, 207-209). The repression is most likely due to the ability of NC2 to inhibit TFIIA and TFIIB binding to TBP at promoter DNA (198-201, 209). NC2 binds to the basic repeat domain of TBP, which overlaps with the TFIIA recognition site (209). Increased levels of TFIIA displace NC2 from TBP-promoter complexes, suggesting a competitive relationship based on steric exclusion between the factors (209). TFIIA also relieves NC2 repression to various degrees at different promoters *in vitro* (151). This relief from repression correlates with the ability of TFIIA to alter the DNase I footprint of NC2-TBP on promoter DNA. TFIIB binds to a region of TBP that is opposite the TFIIA/NC2 binding surface (209) so it is not clear how NC2 blocks TFIIB-TBP binding.

NC2 α and NC2 β contain histone fold structural motifs and dimerize through these domains (200, 201). Purified NC2 is the size predicted for a heterotetramer composed of two NC2 α / β dimers (151, 201). Histone folds were originally characterized in the core histone proteins (210) but are also present in regulatory proteins including TAF_{II}s, TFIIB, and the HAP or CBF activator proteins (211, 212). The fold consists of an extended helix-strand-helix-strand-helix motif, which dimerizes in a head-to-tail orientation [reviewed in (213)]. Mutational analysis of the histone fold region of yeast NC2 α shows that it is critical for NC2 activity, while other domains are dispensable (206). A C-terminal truncation of the last helix in the histone fold of yeast NC2 α causes a partial loss-of-function mutation that compensates for

a mutation in the RNA polymerase II holoenzyme (204). A similar C-terminal truncation in yeast NC2 α (G. Prelich, personal communication) can compensate for the loss of an activator at the *SUC2* gene (206). The yeast and human NC2 homologues show the strongest similarity in the histone fold region, further emphasizing the importance of the region. The genes encoding NC2 α and NC2 β are both essential for cell viability (204, 205).

NC2 functions as a transcriptional repressor at the majority of class II genes *in vivo* (204). The ability of an NC2 mutation to compensate for a mutation in the SRB4 component of the yeast RNA polymerase II holoenzyme, combined with mRNA analysis of the NC2 mutant, indicates that overcoming negative regulation is a general requirement for class II transcription initiation (204). NC2 can affect class III gene expression as well (205, 214).

Little is known about the regulation of NC2 activity. While both NC2 subunits are phosphorylated at serine residues in yeast (C. Wilson, unpublished data), individual serine to alanine mutations in the NC2 α subunit have no apparent effect *in vivo* (C. Wilson and H. Causton, unpublished data). Various double and triple mutations also have no effect. Recombinant human and yeast NC2 repress transcription by human general transcription factors *in vitro* (200, 201, 203, 205). Both purified and recombinant yeast NC2 repress transcription by the RNA polymerase II holoenzyme *in vitro* (V. Myer and C. Wilson, unpublished data). Thus, no role for NC2 phosphorylation has emerged.

MOT1. The 175 kDa Mot1 protein is another effector of RNA polymerase II transcription which targets TBP. *MOT1* is an essential gene that was cloned as a negative effector of pheromone-responsive genes (215). The *mot1-1* mutation suppresses a deletion of the *STE12* activator of pheromone-

responsive genes (215). The Mot1 protein was independently isolated during a purification of yeast TBP (153). Mot1 binds to DNA-bound TBP and releases TBP in an ATP-dependent manner (153, 216). Mot1 inhibits transcription in an *in vitro* transcription system composed of mammalian general transcription factors and yeast TBP (153). Mot1 decreases the commitment of general transcription factors to a particular template in template challenge experiments (153). TFIIA alleviates the effects of Mot1 on TBP binding, transcription, and template commitment (153), suggesting that Mot1, like NC2, competes with TFIIA for TBP. Mot1 contains the characteristic ATPase domain of the Snf2/Swi2 family of conserved nuclear regulatory factors [reviewed in (217)]. Mot1 immunoprecipitates with TBP in a complex distinct from yeast TFIID (218). Mot1 mutations have been alternatively reported to have primarily positive (159) or primarily negative (215, 216, 219, 220) effects on transcription *in vivo*. The reason for this discrepancy is not clear. Since the transcription of a relatively small number of genes have been analyzed in Mot1 mutants, examining total mRNA levels or a larger number of individual genes would be helpful. In the reports proposing a negative role for Mot1, *mot1* mutations increase the basal transcription of a number of genes but have less effect on more highly expressed genes. As is seen with NC2, a mutation in *MOT1* suppresses a UAS-deletion at the *SUC2* promoter (206).

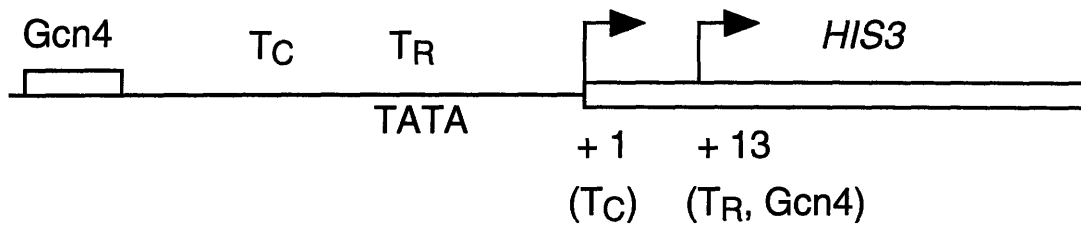
Genetic studies suggest an antagonistic relationship between Mot1 and TBP. The overexpression of TBP is toxic in a recessive mutant, *mot1-1*, presumably because Mot1 is not regulating the increased pool of TBP properly (216). An ATPase-defective dominant *mot1* mutant is suppressed by the overexpression of TBP (216). Because Mot1 remains stably bound to TBP in the absence of ATP, excess TBP may titrate out the mutant Mot1-TBP

complex. Like TFIIA, a *mot1* mutant is synthetically lethal with an *spt3* mutant (159).

B-TFIID. One of several forms of TBP purified from mammalian cells is called B-TFIID. B-TFIID is a 300 kDa complex composed of TBP and a 170 kDa protein (221, 222). Like TFIID, B-TFIID supports RNA polymerase II transcription in a reconstituted *in vitro* system, although B-TFIID is not responsive to several activator proteins (221). Compared to TFIID, transcription with B-TFIID from a subset of promoters shows less stimulation by the addition of TFIIE and TFIIH (223). B-TFIID has ATPase activity (222), reminiscent of the yeast Mot1-TBP complex. However, B-TFIID does not repress any of the promoters that have been tested *in vitro*, although several have very low expression levels when B-TFIID is used instead of TFIID (221, 223). The preincubation of B-TFIID and DNA does not result in stable transcription initiation complexes as measured by template commitment assays (221). This data suggests that B-TFIID, like Mot1, may have a destabilizing effect on TBP at some promoters.

NOTs. A genetic selection for negative effectors of the yeast *HIS3* promoter identified four genes named *NOT1-4* (224, 225). The *HIS3* promoter contains two TATA elements, T_C and T_R , which direct activation from two different transcription start sites, and a binding site for the activator protein Gcn4 (Fig. 2A). T_C supports constitutive expression and does not contain a consensus TATA element whereas T_R contains a canonical TATA element and supports both basal and Gcn4-activated transcription (226). The recessive mutation *not1-2* results in increased transcription from the T_C element, suggesting that Not1 differentially represses that element (Fig. 2B) (224). The *not1-2* mutation also increases the basal and activated expression of a variety

Fig. 2A. Promoter elements of the yeast *HIS3* gene



- Gcn4: equal expression from +1 and + 13
- + Gcn4: 5x increase from +13, no change at +1

Fig. 2B. Differential effects of mutations in regulators

T _C transcription	T _R transcription
Increased by mutations in:	Increased by mutations in:
Not1	Not2 (+ Gcn4)
Not2	Not3 (+ Gcn4)
Not3	Spt6
Not4	NC2 α
Decreased by mutations in:	Decreased by mutations in:
Mot1	Histone H4

of class II genes. Mutations in *NOT2*, *NOT3*, and *NOT4* have similar effects, although several *not2* and *not3* mutations show equal derepression of both T_C and T_R in the presence of Gcn4 (Fig. 2B) (225). The Not1 and Not2 proteins are nuclear and cofractionate on a gel-filtration column (225). Each Not protein interacts with at least one other Not protein by two-hybrid analysis, lending support to the idea of a Not complex (225). Additional evidence of functional interactions comes from genetics; overexpression of *NOT3* or *NOT4* can compensate for *not1* and *not2* mutations (225, 227), a *not2* mutation can suppress a *not1* mutation (225), and a *not4* mutation is synthetically lethal with *not1* or *not2* mutations (227).

NOT1 and *NOT2* were previously cloned as the cell division cycle genes *CDC39* and *CDC36* required for progression through Start (228, 229). *cdc36* and *cdc39* temperature-sensitive mutant strains arrest at the same point in G1 as cells treated with pheromone (228). The mutant strains show increased expression of the pheromone-inducible *FUS1* gene, while other G1 arrest mutants do not (230, 231). The cell cycle arrest and *FUS1* induction phenotypes are dependent on components of the pheromone response signal transduction pathway (230, 231). Diploid cells homozygous for the *cdc36* or *cdc39* mutation arrest asynchronously with respect to the cell cycle, unlike other G1 mutants which show cell cycle arrests regardless of ploidy (228). Altogether, these data indicate the cell cycle arrest phenotype of the mutant *cdc36* or *cdc39* alleles is due to induction of the pheromone response and is dependent on haploid-specific factors.

It is not clear how induction of the pheromone pathway occurs in *cdc36* and *cdc39* mutants. Epistasis experiments suggest that Cdc36 and Cdc39 proteins act at the level of the G protein involved in the pheromone signal transduction pathway (230, 231). The mutant strains have slightly increased

levels of *STE4* mRNA (230), encoding the G β subunit of the heterotrimeric G protein (232). Increased Ste4 protein constitutively activates the pheromone pathway (233). However, mutant strains also show increased expression of *GPA1*, encoding the G α subunit (230). Overexpressed G α decreases the pheromone response in otherwise wild-type cells (233). This suggests that increases in G protein gene expression is not the cause of the pheromone induction seen in the *cdc36* and *cdc39* mutants.

Similar phenotypes are seen in *not4* mutations. Mutant alleles of *NOT4* (also called *MOT2* (227) and *SIG1* (234)) were cloned as suppressors of *ste4* mutations in two independent genetic screens. Like *not1* and *not2*, *not4* mutations induce *FUS1* in the absence of pheromone, and epistasis experiments place Not4 function at the level of the G protein (227, 234). Since there is little biochemical data about the Nots, it is unclear whether the mechanism of Not action is the same in the pheromone signal transduction pathway and the transcriptional regulation seen at *HIS3* and other genes.

A role for Nots in transcription is supported by genetic interactions with other regulators of transcription. An *SPT3* deletion suppresses the *not1-2* mutation (235). At the *HIS3* promoter, the *mot1-1* mutation leads to a decrease in transcription from the T_C element, indicating that Mot1 plays a positive role at the same promoter element where Not1 plays a negative role (Fig. 2B) (235). These data suggest that Spt3 and Mot1 oppose Not1 function in transcriptional regulation, and that TBP is at the center of the battle.

One model proposed to explain the *HIS3* results suggests that a Not-containing protein complex sequesters Spt3, which otherwise binds and stabilizes TBP at the T_C element (235). In the model, Mot1 indirectly boosts T_C expression by increasing the available pool of TBP from elsewhere (presumably canonical TATA elements). But while there is some differential

regulation by these factors at the *HIS3* locus, it may be misleading to generalize from this example. Yeast NC2, which is quite general in its repression of class II genes, shows a strong differential effect on the *HIS3* TATA elements (E. Gadbois, unpublished data) (Fig. 2B). Other regulators, such as Spt6 and histone H4, also show striking differential effects (Fig. 2B) (225).

Recent genetic data suggest that Nots play a more general role in negative regulation than might be concluded from the *HIS3* differential repression studies. Like the general negative regulator NC2, Not1 and Not3 mutants compensate for a mutant SRB4 component of the RNA polymerase II holoenzyme (Chapter 3). Similarly, Not1 and Not2 mutants compensate for a mutant Rpb2 subunit of RNA polymerase II (Chapter 3). The transcriptional defect seen in the *rpb2* mutant is alleviated by the *not2* mutation. These results suggest that Nots negatively regulate a large proportion of class II genes.

Chromatin. Besides the TBP-regulators, there is another class of regulators which is global in activity. This group includes the components and regulators of chromatin structure. The histone proteins assemble chromosomal DNA into nucleosomes, which are bound by other factors and further packaged into higher order chromatin structure [reviewed in (236)].

Several lines of evidence demonstrate the negative effects that nucleosomes can have on transcription. Nucleosome depletion increases the level of transcription of multiple promoters *in vivo* (237, 238). Nucleosomal templates significantly decrease the affinity of activators and TBP for their promoter binding sites and repress transcription initiation *in vitro* (239). The presence of nucleosomes also inhibits the elongation rate of RNA polymerase II *in vitro* (240). While it is hardly surprising that nucleosomes can repress

transcription, there are also cases of transcriptional stimulation by histones (241, 242). Nucleosome positioning at some promoters may bring widely dispersed regulatory elements closer together to activate transcription (243, 244).

Histone and chromatin effects on gene expression are modulated by multiple mechanisms [reviewed in (245-247)]. These mechanisms include the remodeling of chromatin structure, the assembly of chromatin, the acetylation of histones, and regulation through non-histone chromatin components.

There are several ATP-dependent chromatin remodeling activities. As mentioned previously, the Swi/Snf complex within the RNA polymerase II holoenzyme is capable of disrupting nucleosome structure. Other factors which remodel chromatin include the GAGA activator protein and NURF complex in *Drosophila* (248) and the yeast RSC complex (249). Both NURF and RSC contain subunits related to Swi/Snf proteins (249, 250). Genetic data reinforces the link between Swi/Snf proteins and histones; mutations in histones H3 or H4 (251), or decreased levels of the histones H2A or H2B (252), partially alleviate defects caused by *swi/snf* mutations.

Mutations in the *SPT4*, *SPT5*, and *SPT6* genes also suppress *swi/snf* mutations (179). Spt6 interacts with histones, primarily histones H3 and H4, and assembles nucleosomes *in vitro* (253). These results suggest that removing factors which normally assemble nucleosome structure alleviates the need for nucleosome remodeling by Swi/Snf proteins.

The amino termini of the core histones can be acetylated at lysine residues. Histone acetylation correlates with transcriptional activity (254), possibly because the reduction of positive charges in the amino termini weakens DNA interactions. Numerous histone acetyltransferases have

recently been identified (255-261). Several are proteins previously implicated in transcription: human TAF_{II}250 (256) and the transcriptional adaptors p300/CBP (258) and GCN5 (255, 260, 261). Differences in histone acetylase specificities may explain why certain mutations in the N-terminal histone tails only affect the expression of a subset of genes (262).

It has recently been shown by multiple groups that several histone deacetylases form complexes with mammalian proteins homologous to the yeast Sin3 protein (263-268). Sin3, in concert with the histone deacetylase Rpd3, is involved in transcriptional repression of multiple yeast genes (269-272). Mammalian Sin3-histone deacetylase complexes repress transcription through their interactions with hormone receptors (264, 273) and the Mad-Max complex (274). These results reveal mechanisms for transcriptional repression via deacetylation.

Chromatin itself is composed of both histone and non-histone proteins. The non-histone components include factors involved in transcriptional silencing and architectural factors which also influence gene expression. The silent mating loci in yeast (*HML* and *HMR*) and regions adjacent to telomeres are transcriptionally repressed and show similarity to heterochromatin in higher eukaryotes [reviewed in (245)]. Silencing in yeast requires the histones H3 and H4 as well as the Sir3, Sir4, and Rap1 regulators. Sir3 is present at repressed chromosomal regions *in vivo*, and overexpression causes it to spread into neighboring areas (275). The N-termini of the H3 and H4 interact with Sir3 and Sir4 *in vitro* and are necessary for Sir3 positioning on chromosomes (276). Deletions in *sir3* that disrupt the histone interaction lead to loss of silencing *in vivo* (276). Sir3 interacts with Sir4 and Rap1, suggesting that all are structural components of yeast heterochromatin (275, 277). The origin recognition complex (ORC) is also involved in silencing

[reviewed in (278)], possibly indicating a link between replication and silencing. In multicellular organisms, factors such as the *Drosophila* Polycomb-group establish chromatin repression during development [reviewed in (279)].

The high mobility group (HMG) proteins compose a family of architectural chromatin components which affect gene expression. HMG proteins bind and distort DNA, possibly bringing regulatory elements into closer proximity [reviewed in (280)]. Mutations in two yeast HMG homologues, NHPSA/B, result in decreased activation of several inducible genes (281). NHPSA binds to TBP on promoter DNA and stimulates activated transcription *in vitro* (281). Mammalian HMG-1 and HMG-2 also coactivate transcription in an *in vitro* system (282). In a different *in vitro* system, HMG-1 binds to TBP-DNA complexes and inhibits transcription (152). Both TBP-binding and transcriptional repression by HMG-1 in this system can be reversed by increasing amounts of TFIIA (152). Different HMG proteins can have opposing effects through the same factor: in *Drosophila*, HMG1(Y) stimulates activation by NF- κ B (283), while another HMG-1 protein, DSP1, converts NF- κ B to a repressor (284).

These studies of NC2, MOT1, Nots, and chromatin structure indicate that negative regulation is a general component of the landscape that transcription factors must navigate in order to activate gene expression.

My contributions to these projects

Negative regulation. When I began studying transcription in 1994, the yeast RNA polymerase II holoenzyme had recently been purified by Tony Koleske. Other members of the lab were identifying holoenzyme components and characterizing holoenzyme *in vitro* and *in vivo* activities. Craig Thompson had generated conditional alleles of *SRB4* and *SRB6* to demonstrate that the holoenzyme transcribed the majority of class II genes *in vivo*. To identify regulators of holoenzyme activity, I isolated a large collection of spontaneous suppressors of a conditional phenotype of the *srb4-138* allele. My genetic analysis demonstrated that recessive suppressors included alleles of the genes encoding yeast NC2 and Not1 and Not3. I focused on characterizing NC2, while other members of the lab began studying the dominant suppressors, the remaining recessive suppressors, and the *NOT* genes.

The first *SRB4* suppressor that I cloned was *NCB1* (which encodes the NC2 α subunit of NC2). I generated a complete deletion of the gene, demonstrating that *NCB1* is essential for cell viability. I recovered the suppressing allele by gap-repair and sequenced the mutant and wild type alleles. Sequence analysis of *NCB1* showed weak homology to histone H2A. I contacted Fred Winston to investigate the possibility that *NCB1* might be one of the *SPT* genes with phenotypes similar to histones. Winston informed me that *NCB1* had not been cloned as an *SPT*, but had recently been cloned by his former postdoc, Greg Prelich, as a bypass suppressor of an upstream activating sequence. To check whether NC2 α bound to TBP, I collaborated with Joe Reese, who had previously purified the yeast TAF_{II}s by GST-TBP affinity chromatography. Joe Reese checked his eluate fractions with antibodies against NC2 α . Indeed, NC2 α did bind to TBP, and eluted separately from the

peak of the TAF_{II}s. Soon afterwards, Danny Reinberg reported that a TBP-associated negative regulator of transcription, Dr1, was associated with a corepressor, DRAP1. Sequence comparison showed significant homology between DRAP1 and NC2 α . I searched for homologues with the Dr1 amino acid sequence and found a homologous uncharacterized yeast open reading frame. The DRAP1/Dr1 complex had been originally purified by the Roeder lab as NC2, so I named the yeast subunits NC2 α (encoded by *NCB1*) and NC2 β (encoded by *NCB2*). I have since found that an *ncb2* mutant is among the other recessive suppressors of *srb4-138*.

To biochemically characterize yeast NC2, David Chao and I further purified the complex from the TBP column eluate. I confirmed that the two subunits were NC2 α and NC2 β by Western analysis with my antibodies against the *NCB1* and *NCB2* gene products. The yield from this purification was quite low, so I constructed a yeast strain with a flag-tagged NC2 α that David Chao used to purify more NC2. David Chao used this preparation to show that NC2 inhibited transcription by RNA polymerase II holoenzyme in an *in vitro* transcription system.

The *in vitro* experiments with mammalian and yeast NC2 demonstrated its ability to repress transcription, but did not address its *in vivo* relevance. Therefore, I analyzed mRNA levels and specific class II messages in strains containing *srb4-138* and *ncb1-1* mutations. This analysis demonstrated that NC2 is a global repressor of class II gene transcription.

After the identification of *ncb1-1* as a suppressor of *srb4-138*, I assayed a variety of other genes which had been implicated in negative regulation for their presence among my other recessive suppressors. By this method I identified mutant alleles of *NOT1* and *NOT3* genes as additional *srb4-138* suppressors. I confirmed these results by genetic linkage analysis. Tony Lee

found that mutant alleles of *NOT1* and *NOT2* also suppressed a mutation in the Rpb2 subunit of RNA polymerase II, and showed that the defect in mRNA synthesis in the *rpb2* mutant was restored by the *not2* suppressing mutation.

Isolation of a mammalian *SRB* gene and RNA polymerase II

holoenzyme. Soon after the yeast holoenzyme was purified, David Chao became interested in identifying a mammalian holoenzyme. Since a distinct hallmark of the yeast holoenzyme is the presence of SRBs, his discovery of a human EST in the database with homology to yeast *SRB7* promised to be a useful marker in a mammalian holoenzyme purification. I isolated the EST fragment from a human cDNA library, which David Chao and Peter Murray then used to clone the entire human gene. To determine whether the human gene was a functional *SRB7* homologue, I tested the human gene for its ability to complement an *SRB7* deletion in yeast. When this was unsuccessful, David Chao and I made chimaeras of the human and yeast genes which proved to be functional in yeast. I raised antibodies against the human protein which David Chao used in a successful purification of a mammalian holoenzyme. A characterization of the holoenzyme preparation by David Chao, Stephen Anderson, and Jeff Parvin showed that it contains TFIIE and TFIIH and can be responsive to activators.

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Chapter 2

Functional antagonism between RNA polymerase II holoenzyme and global negative regulator NC2 *in vivo*

Summary

Activation of eukaryotic class II gene expression involves the formation of a transcription initiation complex that includes RNA polymerase II, general transcription factors, and SRB components of the holoenzyme. Negative effectors of transcription have been described, but it is not clear whether any are general repressors of class II genes *in vivo*. We reasoned that defects in truly global negative regulators should compensate for deficiencies in SRB4 because SRB4 plays a positive role in holoenzyme function. Genetic experiments reveal that this is indeed the case: a defect in the yeast homologue of the human negative regulator NC2 (Dr1·DRAP1) suppresses a mutation in *SRB4*. Global defects in mRNA synthesis caused by the defective yeast holoenzyme are alleviated by the NC2 suppressing mutation *in vivo*, indicating that yeast NC2 is a global negative regulator of class II transcription. These results imply that relief from repression at class II promoters is a general feature of gene activation *in vivo*.

Introduction

Activation of class II gene transcription in eukaryotes involves the recruitment of a transcription initiation complex which includes the RNA polymerase II holoenzyme (1-6). The yeast RNA polymerase II holoenzyme is a large multisubunit complex containing RNA polymerase II, a subset of the general transcription factors, and SRB regulatory proteins (7-11). Mammalian RNA polymerase II holoenzymes have also been purified, and an SRB7 homologue has been identified as a component of those complexes (12-14).

For some class II genes, regulation appears to involve both positive and negative transcriptional regulators. The negative regulators that have been described include proteins purified for their ability to inhibit transcription *in vitro* (15-21) and genes identified because their products repress transcription from a subset of class II genes *in vivo* (21-29). For example, the human proteins NC1 (15, 16), NC2 or Dr1·DRAP1 (16, 17, 20), and DNA topoisomerase I (18, 19) repress basal transcription *in vitro*. The products of the yeast genes *MOT1* (21-24), *NOT1-4* (25-27), and *SIN4* (28-29) negatively regulate at least a subset of yeast genes *in vivo*. Whether any of these negative regulators are generally employed for class II gene regulation *in vivo* is not yet clear.

The RNA polymerase II C-terminal domain (CTD) and the associated SRB complex have been implicated in the response to transcriptional activators (7-9, 30, 31). Two holoenzyme components, SRB4 and SRB6, have been shown to play essential and positive roles in transcription at the majority of class II genes in *S. cerevisiae* (32). We reasoned that a defect in SRB4 might be alleviated by defects in general negative regulators, and that knowledge of such regulators could contribute to our understanding of the mechanisms involved in gene regulation *in vivo*. Here we show that a

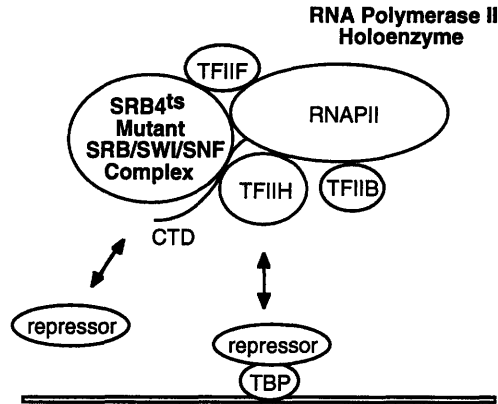
deficiency in yeast NC2 can compensate for the global transcriptional defects caused by mutations in the SRB4 and SRB6 subunits of the RNA polymerase II holoenzyme and that NC2 is a global negative regulator of class II transcription *in vivo*.

Results

Yeast *ncb1-1* is an extragenic suppressor of the *srb4-138* mutation. Since SRB4 plays an essential and positive role in class II transcription, we reasoned that a defect in SRB4 might be alleviated by defects in general negative regulators (Fig. 1A). To identify mutations that compensate for a defect in SRB4, 76 spontaneous extragenic suppressors of the temperature sensitive phenotype of the *srb4-138* allele were isolated. Fifteen of the suppressors were dominant and 61 were recessive. Seven complementation groups were established among the recessive suppressors. One of the recessive suppressing genes was cloned by complementation using a wild-type genomic DNA library and sequenced. The sequence is identical to the open reading frame YER159c, which predicts a 142-amino acid protein with a molecular mass of 15,500 (15.5 K) (Fig. 1B). A search of sequence databases revealed that the predicted protein has 39% identity over 99 amino acids to the NC2 α (DRAP1) subunit of human NC2 (Dr1·DRAP1), which binds to TBP and represses transcription *in vitro* (16, 17, 20, 33-39). The gene encoding the putative yeast NC2 α protein was named *NCB1*. Deletion analysis revealed that *NCB1* is essential for cell viability (data not shown). The mutation present in the suppressing allele, *ncb1-1*, produces a 27 residue C-terminal truncation in the yeast NC2 α protein (Fig. 1B). Since *NCB1* is an essential gene, the truncation mutation must cause a partial functional defect in the NC2 α protein.

Human NC2 consists of two subunits, NC2 α and NC2 β , both of which are necessary for maximal TBP binding and repression of transcription *in vitro* (37, 38). To determine if there is a yeast homologue of the NC2 β subunit, the yeast database was searched with the human NC2 β amino acid

a Genetic Selection for Negative Regulators



b NCB1 gene sequence (yNC2 α /DRAP1 protein)

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1 atggcagatcaagtaccagttacaacacaactaccaccaataaaacctaagaacatgaggtaccacttgatgctggaggagtcagtaggtaacatgggtaccaactcg
M A D Q V P V T T Q L P P I K P E H E V P L D A G G S P V G N M G T N S
109 aataacaacaacgagctaggtgatgtattcgacagaataaaagacacacttccctccggccaaggtaaagaaaataatgcagacagacgaggatagggaaaagtttca
N N N N E L G D V F D R I K T H F P P A K V K K I M Q T D E D I G K V S
-----
217 caagccacgcccgtaatagcgggcaggtccctagagtttttatagcgttattggtgaaaaaaagcggggagatggcaagaggacaaggaaccaagagaataaccgcc
Q A T P V I A G R S L E F F I A L L V K K S G E M A R G Q G T K R I T A
-----
325 gaaataactaaaaaaacaattttaaagcagcaaaaattcgatttcttaaggggaaggtctatgcgtagaagaagcacaacacacccggggaagagagtgacctgagca
E I L K K K T I L N D E K F D F L R E G L C V E E G Q T Q P E E E S A *
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helix 3

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c NCB2 gene sequence (yNC2 β /Dr1 protein)

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1 atggctggagactccgataatgtgtcgcttccaagggtatgttagttatattgttgtgcaaaactcaagctttgatgcccgggtactgagcgggtataactaacttaga
M A G D S D N V S L P K A
-----
109 gaaaactgaatgatccttagcgcggtacaaaagatgatctgaaactggaccaggatttgatggttaccaggatgcaagagaatcatcatcaactccggca
T V Q K M I S E I L D Q D L M F T K D A R E I I I N S G I
-----
217 tagaattcataatgatcctgtcctcgatggcttccgaaatggcggacaacgaggctaaagaaaccatagcggccgagcagtgatcaagcgcgtagaagagttggagt
E F I M I L S S M A S E M A D N E A K K T I A P E H V I K A L E E L E Y
-----
325 ataagagtttataccatttcttagaggaaatattattgaattttaaaggttcccagaaggtgaagaaactagggttccaagttcaagaagtcaggtctctcggaag
N E F I P F L E E I L L N F K G S Q K V K E T R D S K F K K S G L S E E
-----
433 aagagctgctacgacaacaagaggagttgttagacagctcaaggtccagattacaccacaatagtgtatctgatccggttaagtcggaggattcttcttgaatagaag
E L L R Q Q E E L F R Q S R S R L H H N S V S D P V K S E D S S *

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Figure 1. Isolation of putative global negative regulators

(A) Schematic of genetic selection for suppressors of the temperature sensitive *srb4* mutant RNA polymerase II holoenzyme. (B) Sequence of *NCB1* (open reading frame (ORF) YER159c on chromosome V, GenBank accession number U18917). The suppressing allele, *ncb1-1*, was isolated by gap-repair techniques and sequenced. The suppressing mutation, a single base pair deletion at nt 340, is noted in boldfaced type. The deletion results in a frameshift causing a translational stop at nt 347-349, also noted in boldfaced type. Underlined regions indicate homology to α -helices in the histone H2A histone fold (37-39). (C) Sequence of *NCB2* (open reading frame (ORF) D9509.16 on chromosome IV, GenBank accession number U32274) with the first and last nt of the intron sequence noted in boldfaced type. Underlined regions indicate homology to α -helices in the histone H2B histone fold (37-41).

sequence. An open reading frame, D9509.16, was identified that predicts a 146-amino acid protein with a molecular mass of 16,700 (16.7 K) (Fig. 1C). The predicted protein has 37% identity to human NC2 β . The gene, named *NCB2*, contains consensus sequence predicting an intron. Both DNA and cDNA clones containing the coding sequence for *NCB2* were isolated and sequenced, and the intron structure produces a somewhat different amino acid sequence than that predicted by GenBank (Fig. 1C). A mutant allele of *NCB2* was identified as one of the other recessive suppressors of *srb4-138* (Chapter 3).

The human NC2 subunits each contain sequence predicting a histone fold structure (37, 38, 42, 43); the yeast NC2 subunits also exhibit this sequence relationship (Fig. 1B,C) (39). Interestingly, the C-terminal truncation in the *ncb1-1* suppressing allele removes part of the histone fold in the yeast NC2 α subunit (Fig. 1B). Deletions in the human NC2 histone folds have been shown to decrease subunit association, TBP binding, and transcriptional repression (37, 38).

Yeast NC2 binds to TBP and can be purified as a two subunit complex. If the two yeast gene products are genuine homologues of human NC2, they would be expected to copurify as a complex and bind to TBP. To determine whether this is the case, a yeast whole cell extract was subjected to GST and GST-TBP affinity chromatography (Fig. 2A). Western analyses of the column eluates confirmed that both yeast NC2 α and NC2 β proteins were specifically retained on the GST-TBP column. The eluate from the GST-TBP column was further purified over two ion-exchange columns. Silver staining and Western analyses showed that yeast NC2 α and NC2 β coeluted over both columns, and that the proteins appear to be in equal stoichiometry (Fig. 2B and data not shown). Yeast NC2 α is not present in a purified RNA

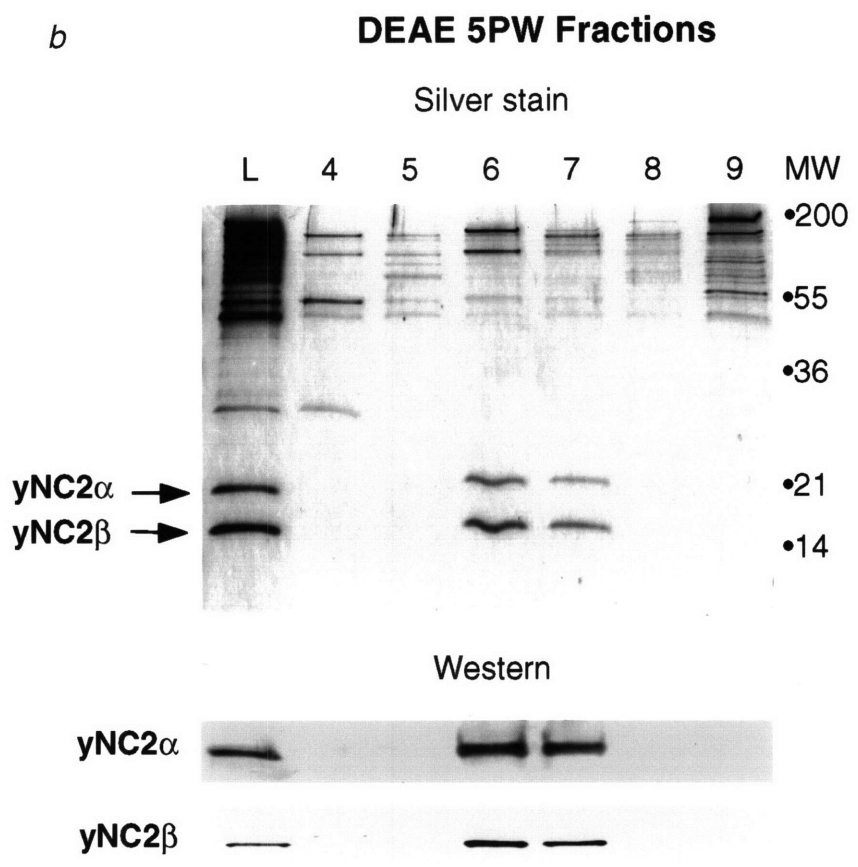
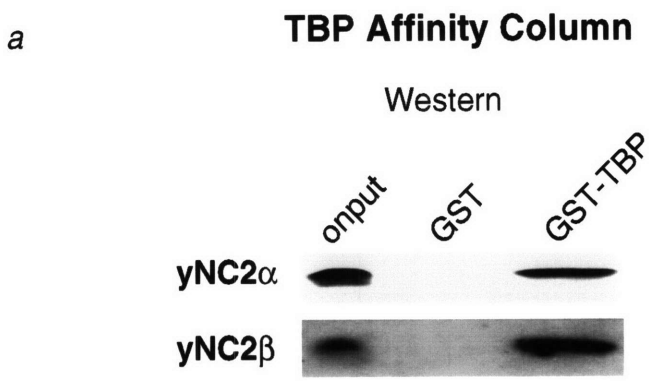


Figure 2. Yeast NC2 binds to TBP and can be purified as a two subunit complex.

(A) Western analyses of TBP column output and eluates with antibodies against yNC2 α and yNC2 β . Bound proteins were eluted with 2 M KCl. (B) Silver stained SDS polyacrylamide gel and Western analyses of fractions from the final step of the purification (DEAE 5PW).

polymerase II holoenzyme preparation, so NC2 is unlikely to be a component of the holoenzyme (Appendix A). These data confirm that the yeast NC2 α and NC2 β proteins are stoichiometric subunits of a complex which can bind specifically to TBP.

Highly purified yeast NC2 inhibits transcription by RNA polymerase II holoenzyme *in vitro*. The observation that a defective form of yeast NC2 can compensate for a weakened RNA polymerase II holoenzyme suggests that yeast NC2 normally functions to repress holoenzyme activity. We tested the ability of purified yeast NC2 to repress transcription by yeast RNA polymerase II holoenzyme *in vitro*. A preparation of yeast NC2 from a strain containing an epitope-tagged NC2 α subunit (Fig. 3A,B) gave us material of higher yield and purity than from the TBP-affinity column. *In vitro* transcription reactions were performed with a yeast *CYC1* promoter template, holoenzyme, and fractions from the final column of this yeast NC2 purification (Fig. 3C,D). Repression of transcription correlated with the peak of yeast NC2 protein. 50% of the maximal inhibition was observed when an equimolar amount of NC2 was added to RNA polymerase II holoenzyme and TBP (Fig. 3D). The repression of RNA polymerase II holoenzyme transcription by yeast NC2 is consistent with the ability of a partial loss-of-function NC2 mutation to suppress a holoenzyme mutation.

NC2 functions at the majority of class II promoters *in vivo*. The observation that loss of NC2 function in yeast cells can compensate for a defect in the SRB4 component of the holoenzyme, together with previous evidence that SRB4 functions globally at class II promoters (32), suggests that NC2 may repress transcription at class II promoters in general. To determine whether yeast NC2 functions at the majority of class II promoters *in vivo*, we investigated whether the shutdown of mRNA synthesis observed in cells

Mono Q Fractions

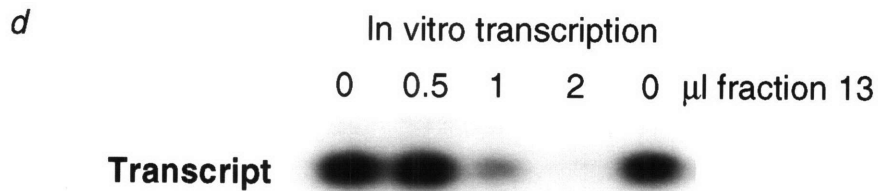
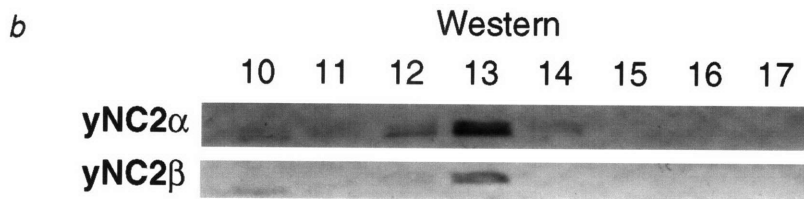
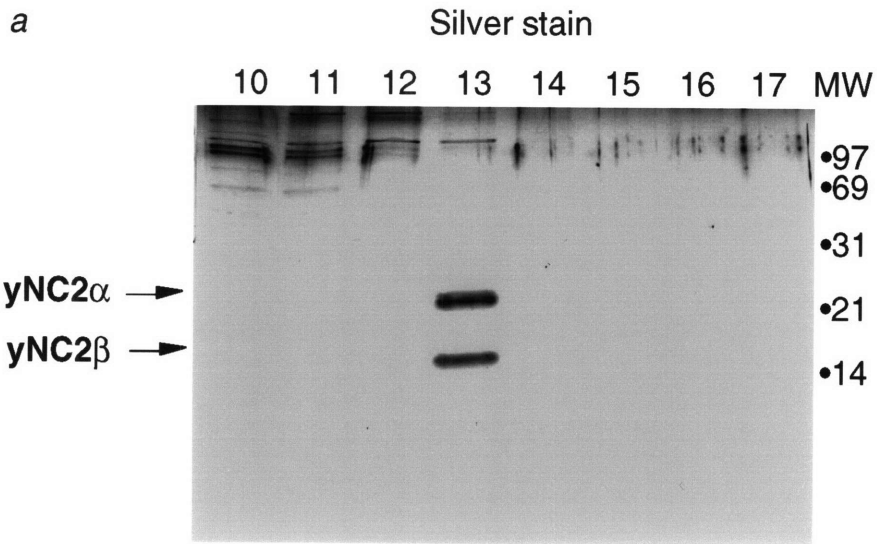


Figure 3. Highly purified yeast NC2 inhibits transcription by RNA polymerase II holoenzyme *in vitro*.

(A) Analysis of NC2 Mono Q fractions by SDS-PAGE and silver staining. (B) Western analyses of Mono Q fractions. (C) Influence of Mono Q fractions on *in vitro* transcription by RNA polymerase II holoenzyme. (D) Inhibition of *in vitro* transcription by RNA polymerase II holoenzyme with increasing amounts of purified yeast NC2. Assuming a molecular weight of 64 kDa for NC2, 0.5 pmol was required for 50% inhibition of an equimolar amount of RNA polymerase II holoenzyme (estimated molecular weight 2,000 kDa) and TBP.

with the temperature sensitive mutant allele *srb4-138* is reversed by the loss of NC2 function (Fig. 4). Upon shifting cells to the restrictive temperature, the growth rate of the *srb4-138* strain was severely reduced, whereas the *srb4-138 ncb1-1* suppressor strain was only modestly affected (Fig. 4A). The levels of poly(A)⁺ mRNA in these cells were measured immediately before and at several times after the shift to the restrictive temperature (Fig. 4B). There was a significant decrease in the mRNA population in the *srb4-138* strain, as observed previously (32). In contrast, there was only a modest decrease in mRNA levels in the *srb4-138 ncb1-1* strain after the temperature shift. Thus, the *ncb1-1* mutation suppresses the general defect in transcription of class II messages caused by the *srb4-138* mutation. Furthermore, the *ncb1-1* mutant in an otherwise wild type background showed 27% higher levels of poly(A)⁺ mRNA compared to the wild type strain under normal conditions (Fig. 4C). This result is consistent with the partial loss-of-function of a class II global negative regulator. S1 analysis of individual class II transcripts confirmed that the decline in specific mRNAs in the *srb4-138* strain is reversed in the *srb4-138 ncb1-1* strain (Fig. 4D). These results, together with previous evidence that NC2 functions as a repressor of multiple promoters tested *in vitro*, argue that NC2 is a general negative regulator of class II gene transcription.

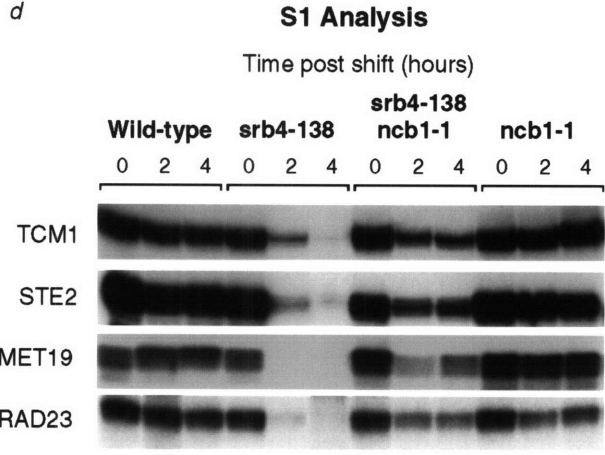
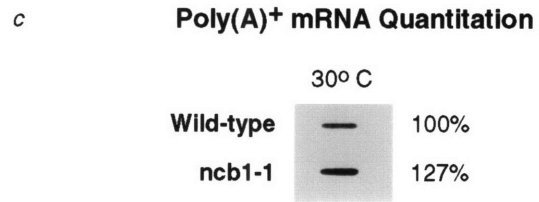
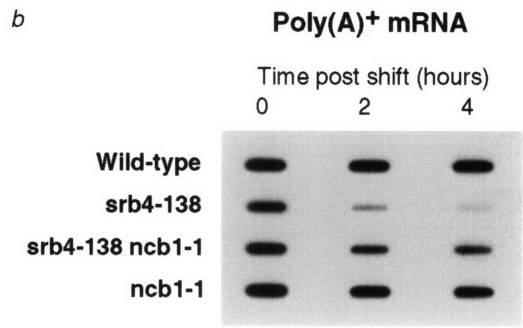
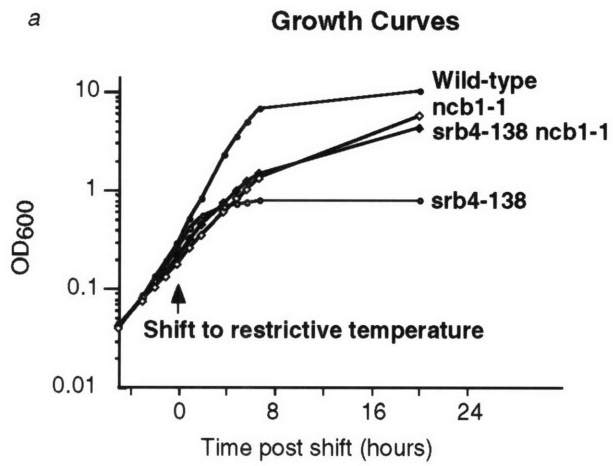


Figure 4. Loss of yeast NC2 function compensates for the global defect in class II gene expression caused by the SRB4 mutant holoenzyme.

(A) *ncb1-1* mutation suppresses the growth defect of the *srb4-138* mutant strain at the restrictive temperature. Growth of wild type (Z579), *srb4-138* (Z628), *srb4-138 ncb1-1* (Z804), and *ncb1-1* (Z805) strains in YPD medium at 30°C and after shifting to the restrictive temperature of 35.5°C. (B) The global decline in mRNA levels at the restrictive temperature in *srb4-138* mutant strain is alleviated by the *ncb1-1* mutation. (C) Global levels of mRNA are increased in the *ncb1-1* strain relative to the wild type strain. (D) The decrease in synthesis of individual class II messages at the restrictive temperature in the *srb4-138* mutant strain is reversed by the *ncb1-1* mutation.

Discussion

Our results indicate that NC2 is an essential and conserved negative regulator of class II gene transcription. These results extend the known negative regulatory effects of NC2 on core RNA polymerase II *in vitro* (16, 17, 20, 33-39) and confirm that this regulator can inhibit transcription by RNA polymerase II *in vivo*.

Since SRB4 has an essential and positive role in transcription at the majority of class II genes in yeast (32), we reasoned that suppressors of a temperature sensitive SRB4 mutant should include negative regulators. In principle, such regulators could repress most class II genes or they could repress SRB4 specifically. Several lines of evidence argue that NC2 acts globally as a repressor of most, if not all, class II genes. NC2 can repress transcription *in vitro* from a wide variety of mammalian, viral, and yeast promoters (16, 20, 36-39). The NC2 suppressor mutation that compensates for reduced class II gene transcription *in vivo* due to loss of SRB4 also compensates for the global defect due to loss of SRB6 (see Appendix A). Loss of function of NC2 in otherwise wild type cells results in increased levels of poly(A)⁺ mRNA. These data do not prove that NC2 regulates all class II genes, but are most consistent with a global role for this repressor.

Mechanism of yeast NC2 repression. Much is already known about the biochemistry of NC2 repression: NC2 binds to TBP on promoter DNA and subsequently inhibits the binding of TFIIA and TFIIB *in vitro* (16, 17, 36-38). NC2 binds to the same basic region of TBP as TFIIA (36), suggesting that NC2 physically blocks TFIIA from binding to TBP.

The other proteins known to have global negative regulatory properties are the histones (44,45), which share notable structural features

with NC2/Dr1·DRAP1. The presence of the histone fold motif in NC2 raises the intriguing possibility of interactions with other histone fold-containing proteins. Histones H2A, H2B, H3 and H4 all contain histone folds (42), as do the yeast HAP3 and HAP5 activator proteins (CBF proteins in mammals) (43) and several TAF_{II}s (40, 41). These TAF_{II}s and histones are able to interact with each other *in vitro* through their histone fold regions (40). Thus, NC2 might introduce a nucleosome-like structure at the promoter, either by itself or with other histone fold-containing proteins.

Transcription activation and relief from repression. The SRB components of the RNA polymerase II holoenzyme contribute to the response to transcriptional activators (7-9). We have shown that a partial loss in NC2 function compensates for deficiencies in SRB4 and SRB6 functions. These results indicate that relief from NC2 inhibition is a required step during transcription initiation at most class II promoters *in vivo*. Evidence consistent with this view has recently emerged from a study of *SUC2* gene regulation. Prelich and Winston (46) isolated yeast mutations that suppress a deletion of the upstream activating sequence in the *SUC2* promoter. The mutant genes that compensated for the absence of *SUC2* activator function included several histones, certain *SPTs*, and other unidentified genes called *BURs* (**B**ypass **U**AS **R**equirement). The *bur6* mutant allele was recently found to be a partial loss-of-function mutation in *NCB1* (47). The observation that *BUR6* is identical to *NCB1* indicates that a loss in NC2 function can compensate for the loss of an activator. These data support the model that activators function to recruit the transcription apparatus, which must overcome negative regulation by NC2 in order to initiate transcription.

Experimental Procedures

Genetic Manipulations. Yeast strains and plasmids are listed in tables I and II, respectively. Details of strain and plasmid constructions are available upon request. Yeast media and manipulation were as described (9). Extragenic suppressors of the temperature-sensitive phenotype of Z628 capable of growth at the restrictive temperature of 36°C were isolated. Dominant and recessive suppressors were identified by mating to Z811 and assaying growth at 36°C on YPD. Complementation groups were established as described (9).

To determine whether the *NCB1* gene is essential for cell viability, the entire coding region was deleted on one of the two chromosomes of a diploid cell, using a single step disruption method (48) and the plasmid RY7136, which carries the deletion allele *ncb1Δ1*. Southern analysis was used to confirm that a single copy of the *NCB1* gene had been deleted. These heterozygous diploid cells were sporulated, and tetrad analysis performed on YPD plates and scored for growth at a variety of temperatures. Spores with the *ncb1Δ1* allele did not produce colonies, indicating that *NCB1* is essential for cell viability.

DNA Methods. DNA manipulations were performed as described (49). PCR amplifications to produce RY7133, RY7134, RY7136, RY7137 and RY7138 were performed with Vent DNA polymerase (New England Biolabs) as described by the manufacturer. The GST fusions were constructed as described (13), and the *ncb1Δ1* allele was constructed as described (50).

Cloning and Sequence Analysis. The genomic clone of *NCB1* was isolated by complementation of Z804 with a wild-type genomic library (50). The wild-type gene was further localized by subcloning fragments of the genomic insert and repeating the screen. The clone with the smallest insert,

RY7135, was sequenced. The genomic clone of *NCB1* was used to confirm the identity of each member of the complementation group and to identify additional members. RY7138 was created from RY7135 *in vivo* by transforming Z804 with linearized RY7135 lacking *NCB1* coding DNA and then isolating the plasmid from a transformant which had repaired the plasmid with the mutant *ncb1-1* sequence from the chromosome (48). *NCB1* and *ncb1-1* were completely sequenced on each strand using DNA from RY7135 and RY7138, respectively. Double stranded sequencing with dideoxynucleotides and Sequenase (US Biochemical) was carried out as described by the manufacturer using T3 and T7 promoter primers and internal oligonucleotide primers. Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (51). The *ncb1-1* mutant allele contained a single base pair deletion at nt (nucleotide) 340, causing a frameshift and a translational stop at nt 347-349 (Fig. 1B). Unlike the RY7135 plasmid, RY7138 did not prevent growth at 36°C when transformed into Z804, indicating that the correct gene was cloned.

Antibodies. Recombinant yNC2 α and yNC2 β proteins were purified for generating polyclonal antibodies in rabbits. Recombinant proteins were derived from *E. coli* containing pGEX-4T-3 (Pharmacia) constructs RY7133 and RY7134 as described (52). The antibodies were used to detect yNC2 α and yNC2 β in Western blots at a dilution of 1:250 or 1:500.

Purification of Yeast NC2. TBP (TATA-binding protein) affinity chromatography was performed as described (53) starting with 1.2 kg of cell pellet. Approximately 60% of the total cellular amount of each NC2 subunit was eluted in 1 M KOAc. 80 ml (3.3 mg) of the 1 M KOAc eluate was dialyzed against buffer T plus 0.003% NP40. The dialyzed sample was applied to a 1 ml

HiTrap SP cartridge (Pharmacia) at 1 ml/min, which was washed with 10 ml of buffer A (20 mM K-HEPES pH 7.6 1 mM EDTA 10% glycerol protease inhibitors) + 100 mM KOAc. Bound proteins were eluted with a 10 ml gradient of Buffer A from 100 mM to 1000 mM KOAc at 0.25 ml/min. Peak NC2 fractions were pooled, frozen in liquid nitrogen and stored at -70°C until use. One half of the peak NC2 fractions (1 ml, 80 ug) was diluted with 2.7 ml Buffer B (20 mM TrisOAc pH 7.8 1 mM EDTA 10% glycerol) and applied to a DEAE 5PW 5/5 column (Toso Haas) at 0.5 ml/min. The column was washed with 5 ml of Buffer B + 100 mM KOAc, and bound proteins were eluted with a 12 ml gradient of Buffer B from 100 to 1000 mM KOAc. The peak of NC2 contained 50 ug total protein. SDS PAGE and silver staining were performed as described (8).

Construction of FLAG-tagged NC2 α Yeast Strain. Plasmid RY7137 was constructed by amplifying the *NCB1* gene (including regulatory sequences) with two sets of overlapping primers to add a FLAG epitope (IBI) to the N-terminus of yNC2 α . The two PCR products were gel purified, combined, and the entire FLAG-tagged *NCB1* gene was amplified with primers adding 5' HindIII and 3' BamHI cloning sites. The final PCR product was cloned into plasmid pUN105 (54). RY7137 was transformed into a Z806, a yeast strain containing the *ncb1 Δ 1* deletion, by plasmid-shuffle techniques (55) to produce Z807. The FLAG-tagged *NCB1* was fully functional and able to complement the *ncb1 Δ 1* deletion.

Purification of FLAG-tagged Yeast NC2 and *in vitro* Transcription

Assays. Yeast strain Z807 was grown in YPD to late log phase and harvested by centrifugation. 500 g of cell pellet was resuspended in 500 ml of 150 mM KOAc 60 mM K-HEPES pH 7.6 3 mM EDTA and protease inhibitors. The mixture was poured slowly into a bath of liquid nitrogen, excess liquid nitrogen was

decanted, and the frozen cells were blended for 4 min. in a Waring blender. The blended cells were stored at -70°C until use. The frozen mixture was thawed at 55°C and centrifuged at 12,000 r.p.m. for 30 min. in a GSA (Sorvall) rotor. One volume (600 ml) of Buffer A + 100 mM KOAc and 300 g of damp-dry BioRex 70 (BioRad) resin were added to the supernatant. After stirring for 2 hours, the BioRex 70 was washed with 1 l of buffer A + 0.1 M KOAc on a Buchner funnel. The washed resin was packed into a 5 cm i.d. column and washed with 0.5 l of buffer A + 0.1 M KOAc at a flow rate of 10 ml/min. Bound proteins were eluted with buffer A + 1 M KOAc. Fractions containing protein (115 ml at 4.1 mg/ml) were pooled, frozen in liquid nitrogen and stored at -70°C until use. 32 ml of BioRex 70 eluate was thawed and mixed with 160 ml of Buffer B + protease inhibitors. The diluted eluate was centrifuged at 12,000 r.p.m. for 30 min. in a GSA rotor. The supernatant was applied to a 2 ml FLAG antibody M2 affinity column (IBI), the column was washed with 100 ml of Buffer B + 150 mM KOAc and 10 ml Buffer B + 50 mM KOAc, and bound proteins were eluted with Buffer B + 50 mM KOAc + 50 mM FLAG peptide. The eluate (8 ml) was filtered through a 0.2 μm filter and applied to a Mono Q PC 1.6/5 column (Pharmacia) at a flow rate of 0.1 ml/min, the column was washed with 1 ml Buffer B + 50 mM KOAc + 1 mM DTT, and bound proteins were eluted with a 2 ml gradient of Buffer B + 1 mM DTT from 50 mM to 2000 mM KOAc. SDS-PAGE, silver staining and Western analysis was as described in Fig. 2 legend. *In vitro* transcription reactions were performed with a yeast CYC1 promoter template as described (56) except that 3' O-MeGTP was added to 40 mM, T1 RNase was omitted, and ethanol precipitations were performed with 400 instead of 600 ml.

Poly(A)⁺ Blots and S1 Analyses. Aliquots of cells were removed from culture at the times indicated, total RNA was prepared, and poly(A)⁺ blots,

quantitation, and S1 protection analysis were carried out as described (32).

Table I. Yeast Strains

Strain	Genotype
Z579	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [pCT127 (<i>SRB4 LEU2 CEN</i>)]
Z628	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [pCT181 (<i>srb4-138 LEU2 CEN</i>)]
Z804	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1</i> [pCT181 (<i>srb4-138 LEU2 CEN</i>)]
Z805	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1</i> [pCT15 (<i>SRB4 URA3 CEN</i>)]
Z806	<i>Mat α ura3-52 his3Δ200 leu2-3,112 ncb1Δ1::HIS3</i> [RY7136 (<i>NCB1 URA3 CEN</i>)]
Z807	<i>Mat α ura3-52 his3Δ200 leu2-3,112 ncb1Δ1::HIS3</i> [RY7136 (<i>NCB1 5' FLAG tag LEU2 CEN</i>)]
Z811	<i>Mat α ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [RY7215 (<i>srb4-138 URA3 CEN</i>)]

Table II. Plasmids

Plasmid	Description
RY7133	<i>NCB1</i> in pGEX-4T-3 (Pharmacia)
RY7134	<i>NCB2</i> (amino acids 13-146) in pGEX-4T-3
RY7135	<i>NCB1</i> (1.3 kb) <i>URA3 CEN</i>
RY7136	<i>ncb1Δ1::HIS3</i> in pBluescript II SK(+)
RY7137	<i>NCB1 5' FLAG tag</i> (IBI) in pUN105
RY7138	<i>ncb1-1</i> (1.3 kb) <i>URA3 CEN</i>

Acknowledgments

We thank R. Roeder, P. Sharp, C. Thompson, A. Hoffmann, T. Lee, H. Madhani, and S. Liao for advice and discussions, J. Madison and E. Shuster for strains and plasmids, L. Ziaugra, E. Jennings and V. Tung for technical assistance, F. Lewitter for assistance with database searches, and G. Prelich, A. Goppelt, M. Meisterernst, J. Kim, and D. Reinberg for sharing data prior to publication. E.L.G. and D.M.C. are predoctoral fellows of the Howard Hughes Medical Institute. J.C.R. is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Research Foundation. This work was supported by NIH grants to R.A.Y.

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Chapter 3

**Functional antagonism between RNA polymerase II holoenzyme and NOT
proteins *in vivo***

Summary

A general feature of gene activation at class II promoters is the relief from repression by negative regulators. We have shown previously that the global negative regulator NC2 antagonizes the RNA polymerase II holoenzyme and that a mutation in the NC2 α subunit alleviates defects in the holoenzyme. We now report that mutations in the NC2 β subunit and in the Not1, Not2, and Not3 proteins can also compensate for holoenzyme mutations. The global defects in mRNA synthesis caused by a defective holoenzyme are reversed by *not* mutations, indicating that Not proteins, like NC2, are global negative regulators. These results demonstrate that there are multiple global negative regulators which must be overcome by the class II transcription machinery.

Introduction

The regulation of class II gene expression requires both positive and negative factors. Transcription initiation in yeast involves the recruitment of the RNA polymerase II holoenzyme, which contains RNA polymerase II, general transcription factors, and SRB proteins [reviewed in (1, 2)]. A conditional mutation in *SRB4* results in a rapid and general decrease in class II transcription (3). A partial loss-of-function mutation in the NC2 α subunit of yeast NC2 (Dr1·DRAP1) suppresses the conditional phenotype of the *srb4* mutation and alleviates the decrease in transcription (4). These results indicate that NC2 is a global negative regulator of class II transcription, and imply that relief of repression is a general feature of gene activation.

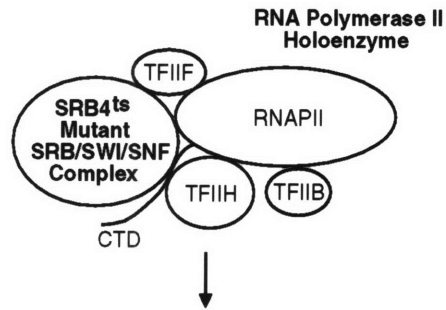
Other negative regulators of transcription have been identified through *in vivo* and *in vitro* experiments [reviewed in (5)]. While many of these factors have been shown to repress the transcription of a number of genes, it is not yet clear whether any are truly global in nature. In order to identify other factors likely to be global negative regulators of class II expression, additional suppressors of the *srb4* mutation were identified. One of the suppressing mutations is in the gene encoding the NC2 β subunit of yeast NC2, indicating that mutations in either subunit of NC2 can alleviate the holoenzyme defect. We also show that mutations in *NOT1* and *NOT3* similarly suppress the *srb4* mutation. Mutations in *NOT1* and *NOT2* suppress a mutation in another holoenzyme component, the second largest subunit of RNA polymerase II. These results suggest that the Nots represent another class of global negative regulators of transcription.

Results

***ncb2-1* is an extragenic suppressor of the *srb4-138* mutation.** The recessive suppressors of the *srb4-138* mutation include at least six different complementation groups (Fig. 1A; Appendix A, Fig. 3). We tested whether *NCB2*, which encodes the NC2 β subunit of yeast NC2, was among the other suppressors. Indeed, complementation group B was determined to represent a suppressing allele of *NCB2* (Fig. 1A, B) by complementation with the wild-type *NCB2* gene. The identity of the suppressing allele was confirmed by plasmid gap repair (data not shown) (6). Thus, mutations in the genes encoding either subunit of NC2 can suppress the *srb4-138* mutation.

***not1-10* and *not3-10* are extragenic suppressors of the *srb4-138* mutation.** Numerous factors in addition to NC2 have been described as negative regulators of transcription. We tested a variety of genes by complementation to ascertain whether they were among the remaining suppressors of the *srb4-138* allele (Appendix A, Fig. 3, 4). Complementation groups C and D were determined to represent suppressing alleles of *NOT1* and *NOT3* (Fig. 1A, B). The *NOT* genes, *NOT1-NOT4*, have been previously implicated in transcriptional repression (7-9) and *NOT1* was originally cloned as *CDC39* (10). The identities of both suppressors were confirmed by genetic linkage experiments (data not shown) (11). A disruption (6) of the *NOT3* gene also suppressed *srb4-138*, indicating that a loss-of-function mutation in *NOT3* could alleviate the holoenzyme defect (data not shown). Interestingly, the *not1* and *not3* complementation groups showed a high degree of unlinked noncomplementation (12), indicating a close functional relationship between the genes (Appendix A, Fig. 3).

a Recessive Suppressors of SRB4 mutant



<u>Complementation Groups</u>	<u># of Isolates</u>
Group A: <i>NCB1</i>	5
Group B: <i>NCB2</i>	1
Group C: <i>NOT1</i>	18
Group D: <i>NOT3</i>	19
Group E:	1
Group F:	4
Non-Complementers	19

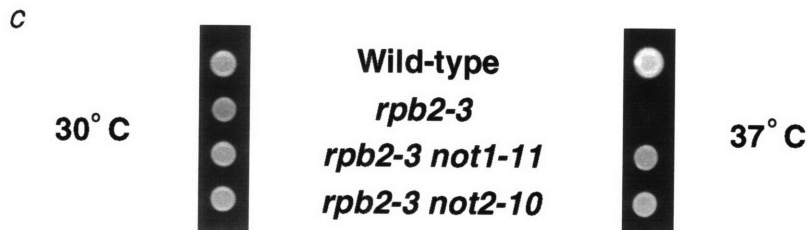


Figure 1. Mutations in multiple negative regulators of transcription compensate for RNA polymerase II holoenzyme mutations.

(A) Recessive suppressors of the *srb4-138* mutation include multiple complementation groups and four known negative regulators of transcription. (B). Growth phenotypes of cells containing the *srb4-138* mutation (Z628) and *srb4-138 ncb1-1* (Z804), *srb4-138 ncb2-1* (Z828), *srb4-138 not1-10* (Z829), and *srb4-138 not3-10* (Z830) mutations compared to wild-type cells (Z579). Cells were spotted on YEPD medium and incubated at 30°C and 36°C for 2 days. (C). Growth phenotypes of cells containing the *rpb2-3* mutation (Z832) and *rpb2-3 not1-11* (Z833) and *rpb2-3 not2-10* (Z834) mutations compared to wild-type cells (Z831). Cells were spotted on YEPD medium and incubated at 30°C and 37°C for 2 days.

***not1-11* and *not2-10* are extragenic suppressors of the *rpb2-2* mutation.**

Since RNA polymerase II is a critical component of the transcriptional machinery (13), we reasoned that defects in polymerase itself might be alleviated by mutations in global negative regulators of class II promoters. The temperature-sensitive phenotype of a mutation in the second largest subunit of yeast RNA polymerase II, *rpb2-3* (14), was used to isolate 173 extragenic suppressors. Thirty-seven were dominant and 136 were recessive. Five complementation groups were established among the recessive suppressors. The suppressing gene from one group was cloned by complementation using a wild-type genomic library, sequenced, and shown to be *NOT2* (Fig. 1C). *NOT2* was originally cloned as *CDC36* (10). Testing of other *NOT* genes revealed that another complementation group contains a mutant *not1* (Fig. 1C). These results, in combination with the identification of *not1* and *not3* as suppressors of *srb4-138*, make a powerful argument for Not proteins as global negative regulators of class II transcription.

Not2 functions at many class II promoters *in vivo*. The genetic suppression of *srb4* and *rpb2* mutant alleles by *not* mutant alleles suggests that Nots, like NC2, may repress transcription at class II promoters in general. We investigated whether the decrease in mRNA synthesis observed in cells with the *rpb2-3* mutation was reversed by the *not2-10* suppressing mutation (Fig. 2). Upon shifting cells to the restrictive temperature, the growth rate of the *rpb2-3* strain was reduced, whereas the *rpb2-3 not2-10* strain was only modestly affected (data not shown). The levels of poly(A)⁺ mRNA in these cells were measured immediately before and at several times after the shift to the restrictive temperature (Fig. 2). There was a significant decrease in the mRNA population in the *rpb2-3* strain. In contrast, there was no apparent decrease in mRNA levels in the *rpb2-3 not2-10* strain after the temperature

Poly(A)⁺ mRNA

Time post shift (hours)

0 2 4 6

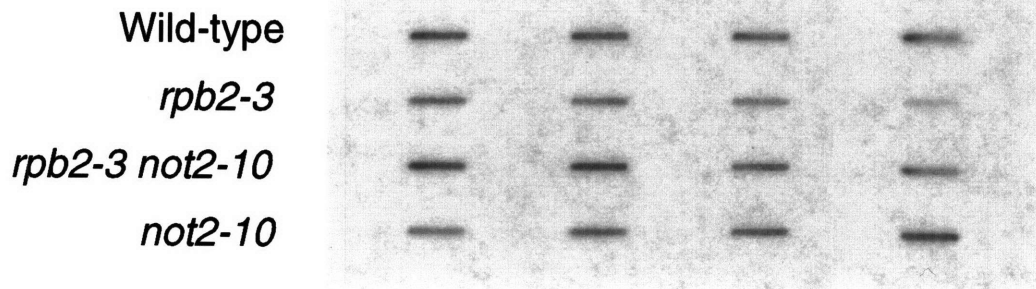


Figure 2. A Not2 mutant compensates for the defect in class II gene expression caused by the Rpb2 mutant holoenzyme.

The decline in mRNA levels at the restrictive temperature in the *rpb2-3* mutant strain is alleviated by the *not2-10* mutation. The levels of poly(A)⁺ mRNA were measured in wild type (Z831), *rpb2-3* (Z832), *rpb2-3 not2-10* (Z834), and *not2-10* (Z835) cells grown in YEPD medium at 30°C and 37°C.

shift. Thus, the *not2-10* mutation suppresses the defect in transcription of class II messages caused by the *rpb2-3* mutation. These results support the model of Nots as general negative regulators of class II gene transcription.

Our results indicate that several global negative regulators of transcription antagonize the RNA polymerase II holoenzyme. Mutations in either subunit of yeast NC2 or in Not1, Not2, or Not3 proteins compensate for mutations in RNA polymerase II holoenzyme components. Like NC2, the mutant Not2 alleviates the class II transcription defect seen in a holoenzyme mutation. Several of the Nots have significant homology with putative proteins from other eukaryotic organisms (T. Lee, unpublished data), suggesting that the Nots are conserved throughout evolution.

Previous genetic data suggests that the Nots are involved in transcription, possibly through some effect on TBP (9). However, other experiments demonstrate a role for Nots in the pheromone response (15-18). Whether the involvement of Nots in signal transduction is an indirect result of alterations in gene repression by Nots is unknown. There have been no mechanistic studies of Not protein activity, and it is unclear how Nots might affect transcription on a global level. The genetic interactions of *NOT1*, *NOT2*, and *NOT3* with RNA polymerase II holoenzyme components supports a central role for Nots in transcription. Biochemical analysis of a purified Not-containing protein complex (V. Myer, unpublished data) may help reveal the mechanism of class II gene repression by the Nots.

Experimental Procedures

Genetic Manipulations. Yeast strains and plasmids are listed in tables I and II, respectively. Details of strain and plasmid constructions are available upon request. Yeast media and manipulation were as described (19).

Extragenic suppressors of the temperature sensitive phenotypes of *srb4-138* and *rpb2-3* were isolated and dominant and recessive suppressors identified as described (4). Complementation groups were established as described (19).

DNA Methods. DNA manipulations were performed as described (20). PCR amplification to produce RY7212 was performed with Vent DNA polymerase (New England Biolabs) as described by the manufacturer.

Complementation analysis. Complementation groups containing mutant alleles of *NCB2*, *NOT1*, and *NOT3* were identified by transforming Z828 with RY7212; Z829 and Z833 with a YCP50 plasmid containing wild-type *NOT1* (gift of M. Collart); and Z830 with a pRS316 plasmid containing wild-type *NOT3* (gift of M. Collart). The resulting strains no longer grew at the nonpermissive temperatures, indicating that the suppression phenotype was reversed by the wild-type *NCB2* and *NOT* genes. The genomic clone of *NOT2* was isolated by complementation of Z834 with a wild-type genomic library (21) as described (4). The clone with the smallest insert, RY7213, was sequenced.

Plasmid gap repair. RY7211 was created from RY7212 lacking *NCB2* coding DNA in Z828 as described (4). RY7214 was created from RY7213 lacking *NOT2* coding DNA in Z834 as described (4).

Genetic linkage analysis. The identities of *not1* and *not3* alleles as suppressors of *srb4-138* were confirmed by genetic linkage analysis. The *URA3* gene was integrated next to the *NOT1* gene in Z836 using Sac1-digested

pES183 (gift of E. Shuster). The resulting strain, Z837 was mated to Z829. The resulting diploid strain was sporulated and 20 tetrads were dissected. Analysis of the resulting spores showed that temperature-sensitive phenotype always co-segregated with the Ura⁺ phenotype, indicating that the suppressing allele was tightly linked to the *NOT1* gene. For *NOT3*, the *URA3* gene was integrated next to the *NOT3* gene in Z836 using Eag1-digested pRS306 with *NOT3* (gift of M. Collart). The resulting strain, Z838 was mated to Z830. The resulting diploid strain was sporulated and 20 tetrads were dissected. Analysis of the resulting spores showed that temperature-sensitive phenotype always co-segregated with the Ura⁺ phenotype, indicating that the suppressing allele was tightly linked to the *NOT3* gene.

Poly(A)⁺ Blots and S1 Analyses. Aliquots of cells were removed from culture at the times indicated, total RNA was prepared, and poly(A)⁺ blots were carried out as described (3).

Table I. Yeast Strains

Strain	Genotype
Z579	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [RY2844 (SRB4 LEU2 CEN)]
Z628	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]
Z804	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]
Z828	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb2-1</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]
Z829	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 not1-10</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]
Z830	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 not3-10</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]
Z831	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb2Δ297::HIS3</i> [RY2129 (RPB2 LEU2 CEN)]
Z832	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb2Δ297::HIS3</i> [RY2349 (<i>rpb2-3</i> LEU2 CEN)]
Z833	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb2Δ297::HIS3 not1-11</i> [RY2349 (<i>rpb2-3</i> LEU2 CEN)]
Z834	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb2Δ297::HIS3 not2-10</i> [RY2349 (<i>rpb2-3</i> LEU2 CEN)]
Z835	<i>Mat α ura3-52 his3Δ200 leu2-3,112 rpb2Δ297::HIS3 not2-10</i> [RY2129 (RPB2 LEU2 CEN)]
Z836	<i>Mat α ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [RY2882 (<i>srb4-138</i> LEU2 CEN)]

Table I. Yeast Strains

Z837	<i>Mat α ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 not1/URA3</i> [RY2882 (<i>srb4-138 LEU2 CEN</i>)]
Z838	<i>Mat α ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 not3/URA3</i> [RY2882 (<i>srb4-138 LEU2 CEN</i>)]

Table II. Plasmids

Plasmid	Description
RY7211	<i>ncb2-1 (1.3 kb) URA3 CEN</i>
RY7212	<i>NCB2 (1.3 kb) URA3 CEN</i>
RY7213	<i>NOT2 (7.9 kb) URA3 CEN</i>
RY7214	<i>not2-10 (7.9 kb) URA3 CEN</i>

Acknowledgments

We thank D. Chao for advice and discussions, M. Collart and E. Shuster for strains and plasmids, and L. Ziaugra for technical assistance. E.L.G. is a predoctoral fellow of the Howard Hughes Medical Institute. This work was supported by NIH grants to R.A.Y.

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Chapter 4

Discussion: General negative regulation

The number and variety of negative regulators of transcription raises two interesting questions. Why does the cell require so many levels of negative regulation? How do the regulators function in relation to each other?

These questions are especially relevant for the negative regulators that function more or less directly through TBP. In spite of the relatively small size of TBP, it is a common target of both positive and negative regulators. NC2 and Mot1 bind to TBP, and *NOT1* and *MOT1* genes show genetic interactions with TBP. These repressors are important factors in gene expression since they are widespread in their repression of class II genes. *NCB1*, *NCB2*, *NOT1*, and *MOT1* are all essential genes, indicating that the proteins are not functionally redundant. Below we consider models for the functions of the individual factors and the relationships between them.

NC2. Extensive biochemistry in yeast and mammalian systems has established that NC2 binds to promoter-TBP complexes and inhibits the binding of TFIIA and/or TFIIB. While this is a plausible model for its *in vivo* repression of many promoters, it does not exclude other possibilities. One alternative model is that NC2 is a specificity factor which binds to TBP bound at non-promoter DNA, thereby blocking improper transcription initiation events. This model seems improbable as a primary function of NC2 since an NC2 mutation, which would increase unblocked TBP bound to non-promoter sites, seems unlikely to suppress an *srb4* mutation. Another possible model is that NC2 regulates a pool of TBP that is not bound to DNA. Although recombinant NC2 can bind TBP in solution in the absence of DNA (V. Myer and C. Wilson, unpublished data), the model of binding TBP on promoter DNA is more attractive for several reasons.

First, NC2 subunits contain histone folds. While it may be coincidental, histone folds have been identified predominantly in transcriptional regulatory proteins, many of which bind to DNA. NC2 is able to bind DNA weakly *in vitro* through its histone fold domains (1), and NC2 extends the DNase I footprint of TBP on promoter DNA (2). Histone fold-containing TAF_{II}s can interact with histones *in vitro* (3), raising the possibility that NC2 also interacts with histones while bound to TBP. Interactions of histone-fold containing proteins like NC2, TAF_{II}s, and HAPs with histones can be imagined to affect gene expression.

The two genetic selections that produced NC2 also support the model of NC2 binding to TBP on the promoter. Mutations in either *NCB1* or *NCB2* suppress the *srb4-138* mutation in the RNA polymerase II holoenzyme. A mutation in *NCB1* (*BUR6*) suppresses a UAS-deletion at the *SUC2* promoter. It is useful to consider these results together with the connection between *SRB4* and activation. SRBs were originally identified as suppressors of a CTD truncation (4). The transcriptional defect in the CTD truncation mutant maps to the UAS regions within several class II genes, including the Gal4 binding site of the *GAL10* UAS (5). The SRB subcomplex of the holoenzyme binds to Gal4-VP16 (6). Analysis of individual SRBs shows that *SRB4* physically and genetically interacts with Gal4 (S. Koh, unpublished data). Altogether, this data suggests that *SRB4* plays an important role in the response to activators. The fact that defects in activation or *SRB4* can be suppressed by NC2 mutations implies that NC2 normally antagonizes the activation process. Activators function in part to recruit holoenzyme (7), which must then overcome repression by NC2, most likely at promoters. Consistent with this view, other suppressors of the *SUC2* UAS-deletion include histones H2A-H2B and H3 (8).

Finally, an NC2 β -Gal4 fusion protein represses transcription *in vitro* from a promoter containing Gal4 binding sites (9). Repression is maximal when NC2 α is present. While this is not a physiologically normal situation, it demonstrates that the presence of NC2 at a promoter can repress transcription.

NOTs. Much less is understood about the function of Not proteins. The differential effects of Not repression at the promoter elements of *HIS3* has led to a model in which Nots are primarily repressors of TATA-less promoters. However, the ability of *not* mutants to suppress *srb4-138* and *rpb2-3* holoenzyme mutations suggests that Nots are global repressors of class II genes. The identification of Not1, Not2, and Not4 as negative effectors of the pheromone response pathway raises the possibility that Nots affect transcription indirectly through a signal transduction pathway. Interestingly, the *MOT1* gene was also cloned as a negative regulator of the pheromone response, yet Mot1 negatively regulates pheromone non-responsive genes and has a defined mechanism of action at promoters. Analysis of a purified Not complex in an *in vitro* transcription system may address whether Nots can repress transcription by directly interfering with the general transcription machinery.

MOT1. Mot1, like NC2, has a straightforward biochemical activity. Most *in vivo* analyses are consistent with the demonstrated ability of Mot1 to remove TBP from promoter DNA and repress transcription *in vitro*. The effect of Mot1 on total mRNA synthesis or a large number of individual genes has not been examined, so it is not clear whether Mot1-mediated repression is widespread among promoters. *mot1* mutants are not among the recessive suppressors of the *srb4-138* mutation (Appendix A, Fig. 4). Likewise, the *mot1-1* mutation does not suppress *srb4-138* (Appendix A). These

negative results do not eliminate the possibility that Mot1 has a general effect on class II transcription, but may reflect mechanistic differences between Mot1 and NC2 or Not proteins.

Mot1 and NC2 are similar in their abilities to bind to TBP on promoter DNA, but their mechanisms of repression are different. NC2 appears to block to transcription initiation while Mot1 apparently removes TBP before initiation can occur. The ability of Mot1 to remove promoter-bound TBP suggests that Mot1 might prevent unregulated reinitiation of the transcription apparatus. This model is consistent with data indicating that mutations in *MOT1* have the greatest effect on genes that are normally expressed at lower levels. Because Mot1 has only been tested in a mammalian *in vitro* transcription system with general transcription factors, it is not known how Mot1 affects yeast general transcription factors or the holoenzyme. It is also not known whether Mot1 can remove TBP from TFIID or NC2-TBP promoter complexes.

A better understanding of Mot1 function might be gained from further analysis of B-TFIID. B-TFIID may represent a significant TBP repository as it was purified from an initial fraction containing >75% of the total cellular TBP (10). While several features of the B-TFIID large subunit make it a likely Mot1 homologue, there are important differences between their *in vitro* activities. Unlike Mot1, B-TFIID supports basal transcription in an *in vitro* system. Since several promoters are only weakly transcribed by B-TFIID, further analysis might reveal promoters which are actually repressed by B-TFIID. As is seen with Mot1, B-TFIID does not promote stable initiation complex formation in template commitment assays.

Interactions of negative regulators. The analysis of *HIS3* gene expression has led others to propose that the Nots, Spt3, and Mot1 have

promoter specificity based on TATA elements. However, the *HIS3* TATA elements do not appear to function independently. The *not1-2* mutant strain requires the presence of the Gcn4 activator protein to show a strong increase in transcription from the T_C element at the permissive temperature, although the T_C element is not Gcn4 responsive in a wild type strain (11). The decrease of transcription from the T_C element in a *mot1-1* mutant requires a wild-type T_R element, yet transcription from the T_R element is not affected by the *mot1-1* mutation (12). Thus, the *HIS3* TATA elements appear to have some influence on each other, making the interpretation of the effects of regulatory mutations difficult. Analysis of additional promoters which are jointly regulated by the Nots, Spt3, Mot1, and NC2 may clarify their respective roles in the cell. The genetic interactions between these factors also highlight the necessity of combining negative regulators for *in vitro* analyses.

Problems in studying negative regulation. There are several problems inherent to the study of negative factors. The isolation of repressors by their ability to inhibit transcription risks identifying non-physiological factors. In addition, genetic approaches can be insufficient for elucidating mechanisms of action. Another problem emerges when trying to define the population of genes affected by a negative effector. The *srb4-138* mutation affects global class II transcription, so it was possible to demonstrate that the *ncb1-1* mutation alleviates that effect. However, only a small increase in class II transcription is detected in an *ncb1-1* mutant in an otherwise wild type cell. It is possible that there is little excess transcriptional machinery available so the loss of a global factor would not result in a larger increase of class II transcription. We were unable to isolate a conditional allele of *NCB1* or rapidly shut down *NCB1* expression, but had we been successful, it could still be argued that the effects of NC2 were not global and that NC2 specifically repressed *SRB4* or some

other gene critical for transcription. Advances in genomic chip technology or current differential display techniques could allow genome-wide surveys of the effects of mutations in transcriptional regulators. These approaches might also be valuable in the identification of promoters that are regulated by individual factors or different combinations of factors. These promoters could then be useful tools for dissecting the interactions and possible specificities of negative regulators both *in vivo* and *in vitro*.

Importance of negative regulation. The complexity of gene regulation reflects the necessity to tailor the expression levels of individual genes to meet different needs in changing environments. Therefore it is not surprising that activators and repressors have intricate relationships and that their relative balances are critical for cell viability. Future genetic and biochemical analyses should help reveal how these factors work in concert to achieve proper gene expression.

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Chapter 5

**A mammalian SRB protein associated with an RNA polymerase II
holoenzyme**

Summary

A large multisubunit complex containing RNA polymerase II, general transcription factors, and SRB regulatory proteins initiates transcription of class II genes in yeast cells¹⁻⁴. The SRB proteins are a hallmark of this RNA polymerase II holoenzyme, as they are found only in this complex, where they contribute to the response to regulators⁴⁻⁸. We have isolated a human homologue of the yeast *SRB7* gene and used antibodies against human SRB7 protein to purify and characterize a mammalian RNA polymerase II holoenzyme containing the general transcription factors TFIIE and TFIIH. This holoenzyme is more responsive to transcriptional activators than core RNA polymerase II when assayed in the presence of coactivators.

Results

A human cDNA clone encoding a protein similar to *Saccharomyces cerevisiae* SRB7 was isolated from a lymphocyte cDNA library by using information derived from expressed sequence tags. The sequence of this human cDNA clone (*hSRB7*) predicts a 144 amino acid protein with a molecular weight of 15.7 kD (Figure 1A). The predicted protein is 35% identical to yeast SRB7 (*ySRB7*) (Figure 1B). We tested *hSRB7*'s ability to functionally substitute for *ySRB7* by determining whether *hSRB7* could complement a complete deletion of the yeast gene. Although full length *hSRB7* failed to complement a *ySRB7* deletion, several chimaeras containing the N-terminus of the human protein and the C-terminus of the yeast protein were able to do so (Figure 1C and D). The complementing chimaera with the largest amount of *hSRB7* contains 57% human sequence.

The yeast SRBs have functional and physical interactions with the RNA polymerase II carboxyl terminal repeat domain, or CTD (reviewed in ref. 4). If the protein encoded by *hSRB7* is a genuine homologue of yeast SRB7, then it should be among a small subset of cellular proteins capable of binding to a recombinant CTD column⁶. Extracts were prepared from yeast, HeLa, and calf thymus and subjected to CTD affinity chromatography (Figure 2A). Western blots of the column eluates confirmed that yeast SRB7 was retained on a CTD column and demonstrated that mammalian SRB7 from HeLa cells and calf thymus was also retained on the CTD column (Figure 2B).

Because the yeast RNA polymerase II holoenzyme can be immunoprecipitated with anti-SRB antibodies, similar experiments were used to investigate whether mammalian SRB7 associates with components of the transcriptional apparatus in crude extracts. Indeed, RNA polymerase II

Figure 1.

(A) Human *SRB7* sequence.

(B) Human and yeast *SRB7* alignment. "|" = identity; ":" = comparison value greater than or equal to 0.5; and "." = comparison value greater than or equal to 0.1. as defined by the program BESTFIT.

(C) Complementation of yeast *SRB7* deletion mutant by *hSRB7-ySRB7* chimaera. Control: Yeast containing *SRB7* deletion covered by *ySRB7* plasmid and indicated construct. Test: Yeast containing *SRB7* deletion and indicated construct.

(D) Complementation by *hSRB7-ySRB7* chimaeras. Chimaeras are represented by normalized bars displaying human *SRB7* sequences in black and yeast sequences in white.

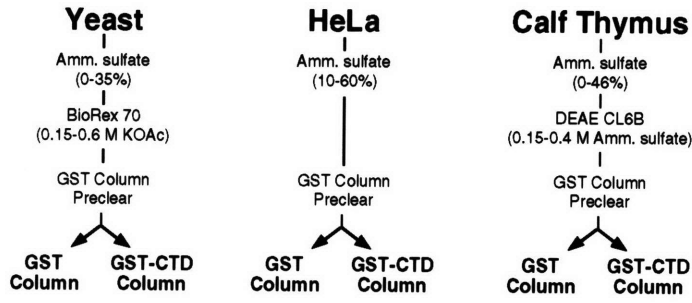
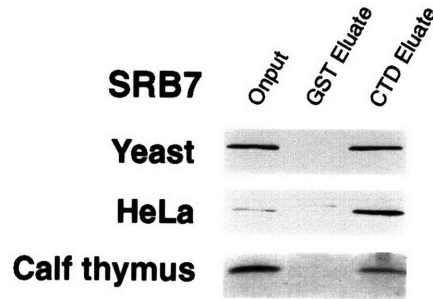
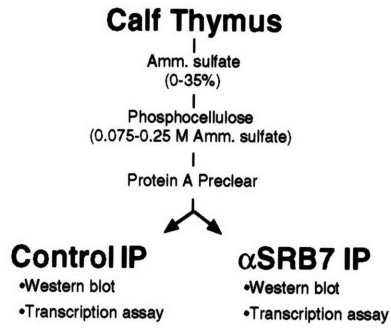
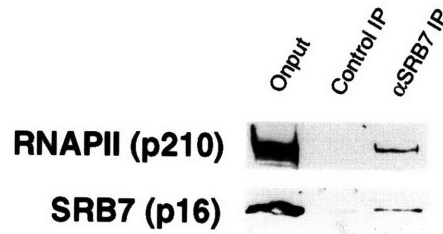
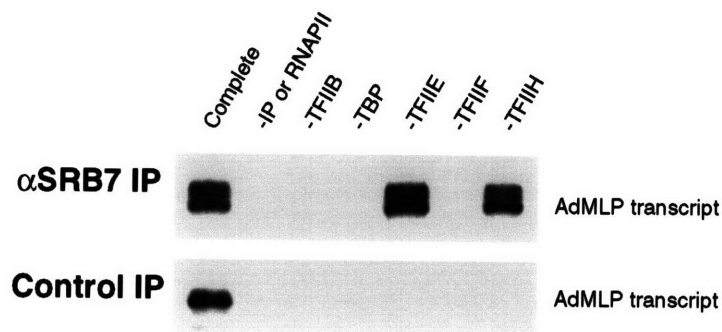
A**B****C****D****E**

Figure 2.

(A) CTD affinity chromatography procedure

(B) Western blots of CTD column eluates. Onputs and eluates were probed with anti-ySRB7 or anti-hSRB7.

(C) Immunoprecipitation procedure

(D) Western blots of anti-hSRB7 immunoprecipitates. Onput and immunoprecipitates were probed with the indicated antibodies.

(E) *In vitro* transcription assays with anti-SRB7 immunoprecipitates.

Complete = reaction containing holoenzyme (α SRB7 IP), TBP, TFIIB, TFIIE, TFIIIF, and TFIIH (*upper* panel) or the same reaction except that core polymerase replaced holoenzyme and a control IP (blocked by peptide) replaced the α SRB7 IP (*lower* panel). For subsequent lanes, the indicated general factor was omitted from the corresponding complete reaction.

was specifically immunoprecipitated by anti-hSRB7 antibody (Figure 2C and D). *In vitro* transcription assays confirmed the presence of RNA polymerase II and revealed the presence of TFIIE and TFIIH activities in the anti-SRB7 immunoprecipitates (Figure 2E). These experiments provide evidence for a mammalian holoenzyme complex containing at a minimum SRB7, RNA polymerase II, TFIIE, and TFIIH.

The complex containing mammalian SRB7 and RNA polymerase II was purified over six columns (Figure 3A). As determined by Western blotting, RNA polymerase II and SRB7 coeluted precisely from the last three columns of the purification. Analysis of material from the last column revealed that SRB7 and RNA polymerase II coeluted with subunits of TFIIE (p56) and TFIIH (p89) (Figures 3B, C and D). The number of coeluting polypeptides present in the SRB7-RNA polymerase II complex is consistent with the complex's estimated size of 2 mDa, as determined by gel filtration chromatography of crude extracts (data not shown). The preparation appears close to purity as defined by coelution of the same set of proteins over two columns. However, *in vitro* transcription results indicate that TFIIE and TFIIH are substoichiometric (Figure 4A), suggesting that a portion of their activities was lost by dissociation or inactivation during purification.

We next compared the responses of purified mammalian holoenzyme and core RNA polymerase II to the activator Gal4-VP16 and the coactivators HMG2^{9, 10} and PC4^{11, 12}. In four independent experiments, the holoenzyme was more strongly inhibited by PC4 and HMG2 in the absence of activator and exhibited a modestly enhanced response to activator in the presence of these coactivators (Figure 4B). Transcription by core RNA polymerase II was mildly inhibited by PC4 and HMG2 (compare lanes 1 and 3) and was stimulated approximately 2-fold by activator (compare ratio of upper

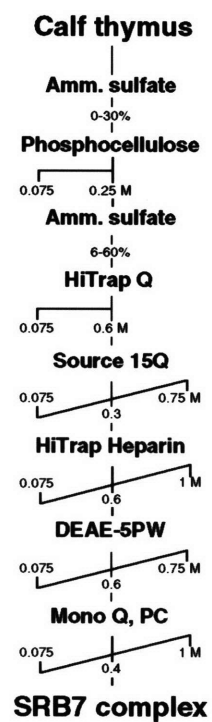
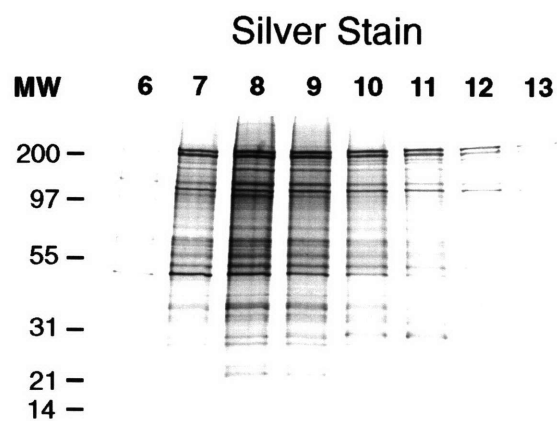
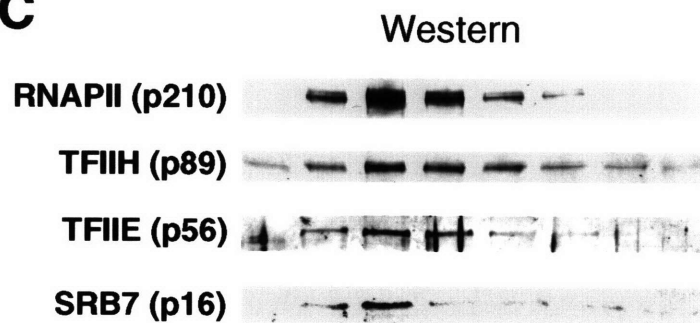
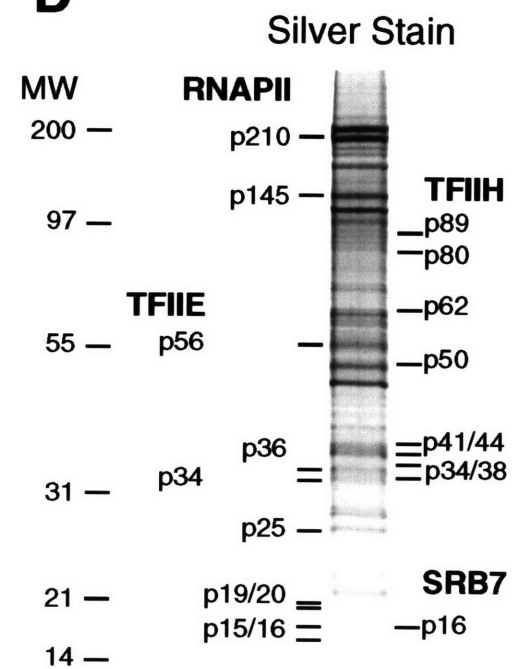
A**B****C****D**

Figure 3.

(A) Procedure for purifying SRB7.

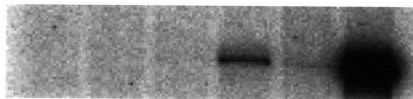
(B) Silver staining of fractions eluted from Mono Q.

(C) Western blotting of fractions eluted from Mono Q. Fractions were probed with the indicated antibodies.

(D) Proposed identities of holoenzyme polypeptides. Bands that correspond in size to RNA polymerase II, TFIIE and TFIIH subunits and mammalian SRB7 are shown.

A**Holoenzyme**

TFIIE	-	-	-	-	+	+
TFIIH	-	-	-	+	-	+
TFIIF	-	-	+	+	+	+
TFIIB	-	+	+	+	+	+
TBP	+	+	+	+	+	+

**AdMLP transcript****B****Core****Holoenzyme**

HMG-2, PC4

Gal4-VP16

	-	-	+	+	-	-	+	+
	-	+	-	+	-	+	-	+

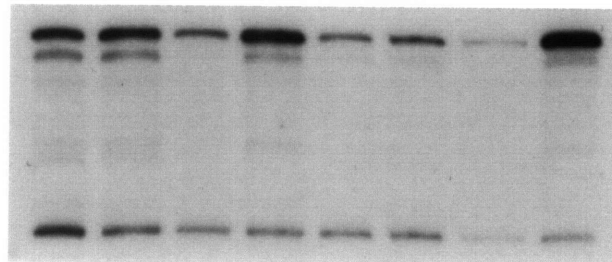
**+ Gal4 sites****- Gal4 sites**

Figure 4.

A). *In vitro* transcription assays. Reactions contained column purified holoenzyme, the indicated general factors, and the Adenovirus Major Late promoter with linear topology.

(B) Response of core RNA polymerase II and column purified holoenzyme to coactivators and activators. Reactions contained core polymerase or holoenzyme, general transcription factors and/or coactivators and Gal4-VP16. The upper transcript is derived from a template containing the Adenovirus Major Late promoter and 3 Gal4 binding sites; the lower transcript is derived from a control template containing the same promoter with no Gal4 binding sites.

and lower bands in lanes 3 and 4). Transcription by holoenzyme was more strongly inhibited by PC4 and HMG2 (compare lanes 5 and 7) and was stimulated approximately 5-fold by the activator (compare ratio of upper and lower bands in lanes 7 and 8). It will be interesting to study the holoenzyme's response to other coactivators and cofactors, such as TBP-associated factors (reviewed in ref. 13), topoisomerase I ¹⁴, Dr1/NC2 ^{15, 16} and PC2 ¹⁷.

We have shown that *hSRB7* shares sequence homology with its yeast counterpart, that *hSRB7-ySRB7* chimaeras functionally complement a yeast *SRB7* deletion, that *hSRB7* is specifically retained by a CTD column, and most importantly, that *hSRB7* associates with a transcriptionally active 2 megadalton complex containing RNA polymerase II and general transcription factors. These results lead us to conclude that *hSRB7* is a genuine homologue of a yeast *SRB* gene and that *hSRB7* is a hallmark component of a mammalian RNA polymerase II holoenzyme. We believe that the yeast RNA polymerase II holoenzyme contains RNA polymerase II, *SRB* proteins and the general factors TFIIB, E, F, and H *in vivo*. Because different holoenzyme purification procedures cause the loss of different subsets of the general transcription factors ^{1,2,18}, it is possible that the forms of holoenzyme purified so far are subcomplexes of a larger entity. In this context, it is not yet clear whether the yeast holoenzyme contains TBP *in vivo*. Similarly, it remains to be determined whether the *in vivo* form of the mammalian RNA polymerase II holoenzyme contains some or all ¹⁹ of the general transcription factors. The isolation of a human *SRB* gene and a mammalian RNA polymerase II holoenzyme provides new tools for investigating these and other issues in transcriptional regulation and extends the holoenzyme paradigm from yeast to mammals.

Experimental Procedures

Cloning of hSRB7. dbEST was screened with XREFdb for expressed sequence tags similar to *ySRB7*²⁰. Overlapping sequences (Genbank accession numbers H08048, R19473, and F13227) were identified as encoding a potential *ySRB7* homologue. An *hSRB7* probe was amplified from a human peripheral blood lymphocyte cDNA library (gift of S. Elledge) constructed in λ YES²¹ by PCR with primers derived from the sequence tags. Vent DNA polymerase (New England Biolabs) was used according to manufacturer's directions for all PCR procedures in this paper. *hSRB7* cDNA was cloned and sequenced²² with the initiating ATG assigned based on homology to *ySRB7*. *ySRB7* and *hSRB7* were aligned with BESTFIT (Genetics Computer Group, Inc.).

Construction of chimaeras. Portions of *ySRB7* and *hSRB7* were amplified by PCR. 18 nt hybridizing to the appropriate region of *ySRB7* were added to the C-term. *hSRB7* primer 5' end. The *hSRB7* N-term. primer and *ySRB7* C-term. primer contained 5' Bgl II sites. PCR products were gel purified, combined, amplified with *hSRB7* N-term. and *ySRB7* C-term. primers, gel purified, and cloned into vector DB20LBgIII's Bgl II site (yeast shuttle vector with 2m, *LEU2*, *ADH1* promoter and terminator, gift of L. Guarente). Plasmids RY7023-RY7031 contain full length *ySRB7* (residues 1-140), *hSRB7*(1-20)-*ySRB7*(21-140), *hSRB7*(1-53)-*ySRB7*(55-140), (*hSRB7*(1-77)-*ySRB7*(82-140), *hSRB7*(1-96)-*ySRB7*(95-140), (*hSRB7*(1-117)-*ySRB7*(129-140), full length *hSRB7*(1-144), *hSRB7*(1-77) with stop after residue 77, *ySRB7*(82-140) with ATG before residue 82.

Complementation by chimaeras. Plasmids containing chimaeras were shuffled into yeast strain Z704 (*MATa ura3-52*, *his3 Δ 200*, *leu2-3,112*, *srb7 Δ 1*

(pCH7: *SRB7 URA3 CEN*)²³. Several representative clones of each strain were tested by streaking or spotting.

Preparation of anti-hSRB7 for Western blotting. Plasmid RY7032 was constructed by amplifying hSRB7 (residues 65-92) with primers adding a 5' Bam HI site and a 3' Sal I site and inserting the PCR product into the corresponding sites of pGEX-4T-3 (Pharmacia). GST-hSRB7 was purified as described²⁴ and used to immunize female New Zealand white rabbits with RIBI adjuvant (RIBI ImmunoChem Research, Inc.) according to manufacturer's directions. Anti-hSRB7 was used to detect SRB7 in Western blots at a dilution of 1:250 or 1:500. Monoclonal antibody 8WG16 was used to detect the largest subunit of RNA polymerase II in Western blots²⁵.

CTD chromatography with yeast SRB7. All subsequent purification procedures in this paper were performed at 4°C. 20 ml BioRex 70 fraction²⁶ was mixed with 9 vol. Buffer A (20 mM K-HEPES pH 7.6, 1 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 0.5 mM pepstatin, 0.15 mM leupeptin, and 1 mg/ml chymostatin + 300 mM KOAc) + 1% Triton X-100. All subsequent buffers contained the same protease inhibitors. The diluted fraction was precleared with a 1 ml GST column and applied to a 1 ml GST or GST-CTD column²⁷. Columns were washed first with Buffer A + 1% Triton X-100, then Buffer A, and eluted with Buffer A + 4 M urea. 10 ml onput and 100 ml each eluate (from 3 ml pool) were TCA precipitated and analyzed by SDS-polyacrylamide gel electrophoresis on 4-20% gradient gels (BioRad) and Western blotting²⁸.

CTD chromatography with human SRB7. 3 ml HeLa whole cell extract²⁹ was chromatographed as above. 25 ml onput was analyzed as above except without TCA precipitation. 0.5 ml each eluate (3 ml total) was analyzed as above.

CTD chromatography with bovine SRB7. An unpublished procedure (L. Strasheim and R. Burgess) was modified extensively. 1 kg frozen calf thymus (Pel-Freez) was placed in a nylon bag (The North Face) and broken with a hammer. Broken pieces were added to 2 l 50 mM Tris-OAc pH 7.8, 10 mM EDTA, 10 mM EGTA, 5% glycerol, 0.2 mM DTT. 300 ml batches were mixed in a Waring blender for 2 min. Batches were pooled, blended for an additional 2 min., and centrifuged (5K, 30 min., RC3B centrifuge (Sorvall)). The supernatant was decanted through Miracloth (CalBiochem), centrifuged and decanted through Miracloth again. After the addition of 29.1 g ammonium sulfate (AS) /100 ml, the extract was stirred for 15 min. and centrifuged (5K, 30 min., RC3B). The supernatant was decanted, and the pellet was resuspended in Buffer D (50 mM Tris-OAc pH 7.8, 0.1 mM EDTA, 5 % glycerol) to a conductivity of 300 mM AS. After the addition of 5.5 ml of 10% polyethylenimine per liter, the extract was stirred for 10 min. and centrifuged (8K, 30 min., GS3 rotor (Sorvall)). The supernatant was decanted, and Buffer D was added to a conductivity of 150 mM AS. 200 ml DEAE Sepharose CL6B (Pharmacia) was added, and the slurry was stirred for 1 hr. The resin was collected by filtration, washed with Buffer D + 150 mM AS and packed into a column (5 cm diam.). Bound proteins were eluted with Buffer D + 400 mM AS. The DEAE eluate, as well as all subsequent column eluates, was frozen in liquid nitrogen and stored at -70°C until use. 15 ml DEAE eluate was chromatographed as above. 10 ml onput and 0.8 ml each eluate (from 3 ml pool) were analyzed as above.

Preparation of anti-hSRB7 antibodies for immunoprecipitations.

Because antisera raised against GST-hSRB7 failed to immunoprecipitate SRB7 from crude extracts, antisera was raised against an anti-hSRB7 peptide. A MAP peptide (QTAINKDQPANPTEEYAQLF, hSRB7 residues 39-58, Research

Genetics) was used to prepare rabbit polyclonal antisera. Antibody was affinity purified according to the manufacturer's directions, except that 1 vol. 1 M NaBorate pH 8.5 was used to neutralize the eluate, which was concentrated in a Centriprep 30 ultrafiltration unit (Amicon).

Preparation of phosphocellulose fraction. Extract from 1 kg calf thymus was prepared as above except that the disruption buffer was 50 mM Tris-SO₄ pH 7.6, 10 mM EDTA, 10 mM EGTA, 5% glycerol, 0.1 mM DTT. After the second centrifugation, AS was added to 30% saturation. The suspension was stirred for 15 min. and centrifuged (5K, 1 hr., RC3B). The supernatant was decanted, and the pellet resuspended in Buffer B (20 mM K-HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 0.1 mM DTT) to a conductivity of 75 mM AS and centrifuged (5K, 10 min., RC3B). The supernatant was decanted and incubated with 0.5 l phosphocellulose (Whatman), precycled according to the manufacturer's directions and equilibrated in Buffer B + 75 mM AS. The slurry was stirred for 1 hr., collected by filtration, washed with Buffer B + 75 mM AS, and packed into a column (5 cm. diam.). Bound proteins were eluted with Buffer B + 250 mM AS.

Immunoprecipitations and *in vitro* transcription. 100 ml phosphocellulose eluate was mixed with 200 ml Buffer B + 0.1% NP-40, incubated with 5 ml protein A-Sepharose (Pharmacia) for 1 hr, and centrifuged (5 min., microcentrifuge). For anti-SRB7 immunoprecipitations, the supernatant was removed and incubated with 5 ml protein A-Sepharose, 10 mg irrelevant MAP peptide, and 1.5 mg affinity purified anti-SRB7 peptide antibody for 2 hr. In control immunoprecipitations, 10 mg hSRB7 blocking peptide was substituted for irrelevant peptide. Immunoprecipitates were washed four times with 0.5 ml Buffer B + 50 mM AS + 0.1% NP-40 and

analyzed as above. *In vitro* transcription assays were performed as described 23.

Purification of mammalian holoenzyme. Preparation of phosphocellulose fraction from 1 kg calf thymus was as described for immunoprecipitations. After adding AS to 70% saturation and 15 min. stirring, the suspension was centrifuged (15K, 15 min., GSA (Sorvall)). The supernatant was decanted, and the pellet was resuspended in Buffer C to a conductivity of 75 mM AS and centrifuged (10K, 10 min., GSA). The supernatant was decanted and loaded at 1 ml/min. to three 5 ml HiTrap Q columns (Pharmacia) connected in series. The column was washed at 2 ml/min. with 100 ml Buffer C + 75 mM AS. Bound proteins were eluted with Buffer C + 600 mM AS. Pooled fractions were diluted with 10 vol. Buffer C, centrifuged (10K, 10 min., GSA), and applied at 2 ml/min. to a 25 ml Source 15Q column. The column was washed with 75 ml Buffer C + 75 mM AS. Bound proteins were eluted with a 180 ml gradient (75 to 1000 mM AS). Fractions containing SRB7 were diluted with Buffer B to a conductivity of 75 mM AS and centrifuged (10K, 10 min., GSA). The supernatant was applied at 1 ml/min. to two 5 ml Heparin HiTrap columns (Pharmacia) connected in series. The column was washed with 20 ml Buffer B + 75 mM AS. Bound proteins were eluted with a 90 ml gradient (75 to 1000 mM AS). Fractions containing SRB7 were dialyzed against 1 l of Buffer C + 25 mM AS + 0.01% NP-40 in a Spectra/Por CE 100 kD MWCO dialysis bag (Spectrum) for 2.5 hr. After Buffer C+0.01% NP-40 was added to a conductivity of 75 mM AS, the sample was centrifuged (8K, 10 min., SS-34 rotor (Sorvall)). The supernatant was decanted and applied at 0.25 ml/min. to a DEAE-5PW 7.5X7.5 column (Toso Haas). The column was washed with 10 ml Buffer C + 75 mM AS. Bound proteins were eluted with a 20 ml gradient (75 to 1000 mM AS).

Fractions containing SRB7 were pooled and dialyzed against 1 l of Buffer C + 0.01% NP-40 for 4 hr. After Buffer C + 0.01% NP-40 was added to a conductivity of 75 mM AS , the sample was filtered through a 0.2 mm filter. The filtrate was applied at 0.2 ml/min. to a Mono Q PC 1.6/5 column (Pharmacia). The column was washed with 2 ml Buffer C + 75 mM AS + 0.01% NP-40. Bound proteins were eluted at 25 ml/min. with a 2 ml gradient (75 to 1000 mM AS).

Silver staining and Western analysis of purified holoenzyme. Silver staining of purified holoenzyme was performed as described ⁶. For Western blotting, rabbit polyclonal anti-TFIIH p89 and anti-TFII E p56 (gifts of J. Kim, B. Shykind, P. Sharp) were used at a dilution of 1:500. 3 ml each fraction was analyzed. Identities of holoenzyme polypeptides were assigned based on published compositions of core RNA polymerase II ³¹, TFIIH ³², and TFII E ³³.

***In vitro* transcription with purified holoenzyme.** Transcription reactions containing TFIIA, TFIIB, TFIID, TFII E, TFIIF, and TFIIH were performed as described ³⁰. Holoenzyme was the peak fraction from the Mono Q column. Protein preparations for all of the basal factors used here have been shown to be free of cross-contamination ³⁴. HMG2 (30 ng/reaction) and PC4 (50 ng/reaction) were titrated for optimal activation.

Acknowledgements

We thank Fran Lewitter for assistance with database searches; Steve Elledge for a phage library; Lenny Guarente for a yeast expression plasmid; Lee Strasheim and Dick Burgess for an unpublished RNA polymerase II purification protocol; and Jae Kim, Ben Shykind, and Phil Sharp for antibodies and purified transcription factors. We also thank Gerry Fink, Phil Sharp, Joan Conaway, Ron Conaway, Danny Reinberg, Robert Roeder, and Ueli Schibler for advice and stimulating discussions. J.D.P. thanks Dr. Ramzi S. Cotran for his support. D.M.C. and E.L.G. are predoctoral fellows of the Howard Hughes Medical Institute. S.F.A. was supported by an institutional training grant from the NIH. This work was supported by NIH grants to R.A.Y.

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Appendix A

Extragenic suppressors of an *srb4* conditional mutation

Summary

This appendix provides additional details about the suppressors of the *srb4-138* conditional phenotype.

Figure 1. Categorization of *srb4-138* Suppressors

- 185 Original isolates:
68 from day 2
95 from day 3
22 from day 4
- 106 Strong
70 Weak
9 Petite
- Among strong suppressors:
76 Extragenic
17 Intragenic
13 Intermediate phenotype
- Among extragenic suppressors:
15 Dominant
61 Recessive
- Recessive complementation groups:

Complementation group:	Number of isolates:
Group A: <i>ncb1</i>	6
Group B: <i>ncb2</i>	1
Group C: <i>not1</i>	18
Group D: <i>not3</i>	19
Group E:	1
Group F:	4
Non-complementers	19
Untested	4

Experimental Procedures

Selection for *srb4-138* suppressors. 200 2 ml YPD cultures of the yeast strain Z628 were grown overnight at 30°C and plated at a density of 3×10^6 cells/plate and placed at 36°C. Suppressors arose at a frequency of 1 in 2×10^6 cells. Only one suppressor was picked from each plate. Petite suppressors were identified by their inability to grow on 3% glycerol.

Figure 2. *srb4-138* Dominant Suppressor Identification Numbers:

2
11
19
21
23
31
33
36*+
43*+
49*+
56
65
76*+
91
135
162

* Indicates most dominant suppressors

+ Can not bypass requirement for *srb4-138*
(John Wyrick, unpublished data)

Figure 3. *srb4-138* Recessive Suppressor Identification Numbers:

Group A: <i>ncb1</i>	Group B: <i>ncb2</i>	Group C: <i>not1</i>	Group D: <i>not3</i>	Group E: ?	Group F: ?
73	115*	4	1	14	95*
88*+		13	4	(74)	
114		14	13	(83)	
121		21	14	(90)	
174		(22)	22	100*	
67		61	61	102	
		62	62	153	
		66	66	(177)	
		74	(70)		
		80*	71		
		86	74		
		102	83		
		108	86		
		(109)	90		
		122	94*		
		153	102		
		177	109		
			153		
			177		

Group G:	Noncomplementers	Untested
75	25	63
92	57	85
93*	78	104
(118)	79	171

82
96
97
106

* Indicates founding member of group
Groups E and G: switched mating type and used to establish complementation groups. Groups A, B, C, D, F: used for cloning or testing genes

113
116
124
131
138

Bold type indicates unlinked noncomplementation between group

143
145
159

() Weakly complements

160
173

+ Cannot bypass requirement for *srb4-138*

Figure 4. Genes not represented among recessive suppressors.

Group E:	Group F:	Group G:	Noncomplementers	Untested
<i>ncb1</i>	<i>ncb1</i>	<i>ncb1</i>	<i>ncb1</i>	<i>ncb2</i>
<i>ncb2</i>	<i>ncb2</i>	<i>ncb2</i>	<i>ncb2</i>	<i>not2</i>
<i>not1</i>	<i>not1</i>	<i>not1</i>	<i>not2</i>	<i>hta1-htb1</i>
<i>not2</i>	<i>not2</i>	<i>not2</i>	<i>hta1-htb1</i>	<i>hht1-hhf1</i>
<i>not3</i>	<i>not3</i>	<i>not3</i>	<i>hht1-hhf1</i>	<i>mot1</i>
<i>srb2-11</i>	<i>sin1</i>	<i>sin1</i>	<i>mot1</i>	<i>sgv1</i>
<i>rpb1,2</i>	<i>spt4</i>	<i>spt4</i>	<i>sgv1</i>	
<i>tbp</i>	<i>spt5</i>	<i>spt6</i>		
<i>sin1</i>	<i>spt6</i>	<i>spt10</i>		
<i>sgv1</i>	<i>spt10</i>	<i>spt16</i>		
<i>ada1-4</i>	<i>spt16</i>	<i>hta1-htb1</i>		
<i>swi2</i>	<i>hta1-htb1</i>	<i>hht1-hhf1</i>		
<i>gal11</i>	<i>hht1-hhf1</i>	<i>mot1</i>		
	<i>mot1</i>	<i>sgv1</i>		
	<i>sgv1</i>			
	<i>ubr1</i>			
	<i>tfg1</i>			
<hr/>				
57,63,70,143:	143:		57,63,70:	
<i>ncb1</i>	<i>rpb1,2</i>		<i>srb2-11</i>	
<i>ncb2</i>	<i>tbp</i>			
<i>not2</i>	<i>ada1-4</i>			
<i>hta1-htb1</i>	<i>sin1</i>			
<i>hht1-hhf1</i>	<i>swi2</i>			
<i>mot1</i>	<i>gal11</i>			
<i>sgv1</i>				

Experimental Procedures

Testing recessive suppressors with candidate genes. A variety of candidate genes were transformed on plasmids into *srb4-138* suppressor strains and tested for their ability to reverse the suppression phenotype at 36°C.

Additional *ncb1-1* Suppression Information

***ncb1* truncation mutations suppress *srb6-107* and Δ UAS deletion mutations, but do not suppress an *rpb1-1* mutation.** The conditional *srb4-138*, *srb6-107*, and *rpb1-1* mutant strains cause similar global transcriptional phenotypes¹. To determine whether *ncb1-1* is able to suppress *srb6-107* or *rpb1-1* mutations, *ncb1-1* on plasmid RY7138 was introduced into cells containing *srb6-107* or *rpb1-1* and the chromosomal copy of *NCB1* was deleted using standard procedures². The *ncb1-1 srb6-107* strain, Z808, grew at the restrictive temperature of 36°C, demonstrating that *ncb1-1* suppresses the *srb6-107* mutation. The *ncb1-1 rpb1-1* strain, Z809, did not grow at 36°C, demonstrating that *ncb1-1* does not suppress the *rpb1-1* mutation. Thus, suppression of these holoenzyme mutations by *ncb1-1* is limited to *srb4-138* and *srb6-107*.

***not1* and *mot1* mutant alleles which do not suppress *srb4-138*.** The Not proteins and Mot1 negatively regulate a subset of yeast genes *in vivo*³⁻⁶. To test whether certain mutant alleles in *NOT1* and *MOT1* are able to suppress the *srb4-138* mutation, the conditional alleles *not1-1* and *mot1-1* were introduced into the *srb4-138* strain Z628. Plasmid pES183 (a gift from E. Shuster) containing the *not1-1* allele was integrated using standard procedures² into Z628, creating Z810. Z810 was unable to grow at the restrictive temperature of 36°C, demonstrating that *not1-1* does not suppress the *ncb1-1* mutation. Yeast strain JMY298 (a gift from J. Madison) containing a *mot1-1* mutation was mated to the *srb4-138* strain Z628. The heterozygous diploid cells were sporulated, and tetrad analysis performed. The resulting haploid cells containing *mot1-1* and *srb4-138* alleles were unable to grow at

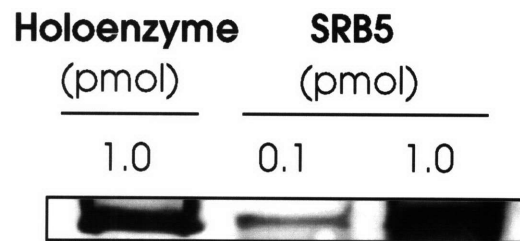
the restrictive temperature of 36°C, demonstrating that *mot1-1* does not suppress the *srb4-138* mutation.

***ncb1-1* suppression of *srb4-138* is not allele-specific.** Additional conditional mutant alleles of *SRB4* (C. Thompson, thesis) were introduced into Z804 by plasmid shuffle⁷ to test *ncb1-1* suppression. *ncb1-1* was able to suppress the conditional phenotypes of all mutants tested, which included *srb4-127*, *srb4-134*, and *srb4-143*. Sequence analysis of 60% of *srb4-127*, *srb4-134*, and *srb4-143* revealed 6, 11, and 4 amino acid changes respectively. Sequence analysis of 95% of *srb4-138* revealed 9 amino acid changes. Thus, *ncb1-1* suppression of *srb4-138* is not allele-specific and all conditional alleles are heavily mutagenized.

***ncb1-1* does not bypass the requirement for *srb4-138*.** *NCB1* and *SRB4* are both essential genes. To determine whether the mutant NC2 α alleviated the need for the mutant *SRB4* protein, the yeast strain Z839 was constructed. Z839 was unable to grow without the plasmid containing the *srb4-138* gene, as assayed on SC 5-FOA⁸ media at both the permissive and restrictive temperatures. Thus, the *ncb1-1* allele does not bypass the need for the *srb4-138* allele.

Quantitative Western Analysis

anti-SRB5:



anti-yNC2 α :

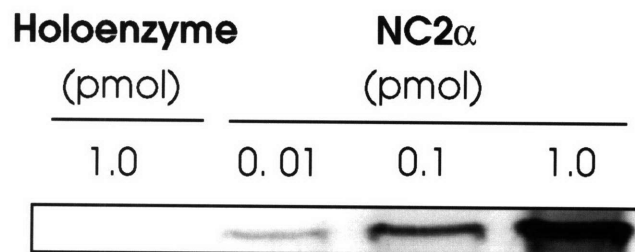


Figure 6. Yeast NC2 α is not a subunit of the RNA polymerase II holoenzyme.

The identification of *ncb1-1* as a suppressor of an *SRB* mutation indicates a functional interaction between yeast NC2 α and the RNA polymerase II holoenzyme. Since it is formally possible that yeast NC2 α is a subunit of the holoenzyme, we used quantitative Western analysis to determine whether yNC2 α could be detected in purified holoenzyme. Known amounts of purified RNA polymerase II holoenzyme⁹ and recombinant yNC2 α and SRB5 proteins were probed with anti-yNC2 α and anti-SRB5 antibodies. SRB5 is a standard we have used previously to quantitate holoenzyme subunits^{10,11}. We did not detect any yNC2 α in purified RNA polymerase II holoenzyme.

Table I. Yeast Strains

Strain	Genotype
Z628	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3</i> [pCT181 (<i>srb4-138 LEU2 CEN</i>)]
Z804	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1</i> [pCT181 (<i>srb4-138 LEU2 CEN</i>)]
Z808	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb6Δ1::hisG ncb1Δ1::HIS3</i> [pCT206 (<i>srb6-107 LEU2 CEN</i>)] [RY7138 (<i>ncb1-1 URA3 CEN</i>)]
Z809	<i>Mat a ura3-52 his3Δ200 leu2-3,112 rpb1-1 ncb1Δ1::HIS3</i> [RY7138 (<i>ncb1-1 URA3 CEN</i>)]
Z810	<i>Mat a ura3-52 his3Δ200 leu2-3,112 not1-1 srb4Δ2::HIS3</i> [pCT181 (<i>srb4-138 LEU2 CEN</i>)]
Z839	<i>Mat a ura3-52 his3Δ200 leu2-3,112 srb4Δ2::HIS3 ncb1-1</i> [RY7215(<i>srb4-138 URA3 CEN</i>)]

Table II. Plasmids

Plasmid	Description
RY7138	<i>ncb1-1 (1.3 kb) URA3 CEN</i>
RY7215	<i>srb4-138 URA3 CEN</i>

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