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Characterization of the interactions within the CCR4 -NOT complex and the interactions between ADR1 and TFIID components

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CHARACTERIZATION OF THE INTERACTIONS WITHIN THE CCR4-NOT COMPLEX AND THE INTERACTIONS BETWEEN ADR1 AND TFIID COMPONENTS

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DISSERTATION

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ABSTRACT

CHARACTERIZATION OF THE PROTEIN INTERACTIONS WITHIN THE CCR4-NOT COMPLEX AND THE INTERACTIONS BETWEEN ADR1 AND TFIID COMPONENTS

By

Yongli Bai

University of New Hampshire, May, 2000

The mechnisms of transcriptional regulation are well conserved from yeast to human cells. Most genes are regulated at the transcriptional level, particularly, at the transcription initiation step. In Saccharomyces cerevisiae at least one transcriptional activator, ADR1, and one transcription complex, CCR4-NOT, participate in the activation of the ADH2 gene. The core $1X10^6$ dalton CCR4-NOT complex consists of CCR4, CAF1, the five NOT proteins (NOT1-NOT5), and three other uncharacterized proteins. Deletion of NOT3, NOT4, or NOT5 did not affect the association of CCR4 with CAF1 in the gel filtration analysis. In contrast, NOT2 inactivation caused partial destabilization of the CCR4-NOT complex. In addition, deletion or mutation in the NOT1 N-

terminal resulted in the dissociation of CCR4 and CAF1 from the complex. TFIID functions in the first step in initiating transcription by binding the TATA-box. To investigate interactions of ADR1 with TFIID in detail, a series of binding assays between ADR1 transcriptional activation domains (TADs) and TFIID components were conducted to identify the individual TAF proteins that interact with ADR1 directly. Multiple TAF proteins were found to bind three of the four TADs of ADR1. TAF150/TSM-1, TAF130/145, TAF60 and TAF61/68 proteins were found to bind TADIV. The region of TAF150 that bound TADIV was located to a nonconserved region (residues 744-1100) while the sequence of TAF130 that alone is capable of binding TADIV was localized to an essential region (residues 668-865) that is well conserved among TAF130 homologs. The physiological roles of these TAF-ADR1 interactions were further investigated using S1 nuclease analysis. An intact TFIID complex was shown to be required for ADH2 gene expression. Individual mutations in TAF150, TAF61, and TAF60 were also shown to reduce ADH2 transcription. Therefore, just as other activators display multiple physical interactions with core transcriptional components, ADR1 also displayed multiple apparently redundant interactions. These biochemical

interactions may benefit cells through redundancy and/or may function in a sequential or conditional way in response to distinct signal transduction pathways.

General Introduction

Baker's yeast, Saccharomyces cerevisae, has proven to be an excellent model system for studying cellular processes. It is a single cell eukaryote with a short life cycle, and its whole genome has been sequenced. In addition, its genetics and DNA recombination are well characterized. The diploid and haploid yeast life cycle facilitates the isolation of both dominant and recessive mutations. It also has unique advantages for meiotic analysis, such as easy triggering of sporulation and monitoring of landmark events. The spores are associated in an ascus, making it easy to reconstruct the recombinant and segregation events (Norbeck et al 1997). More importantly, the mechanisms of most cellular events are conserved from yeast to human, including that of gene expression.

Eukaryotic gene expression is regulated at multiple levels. Chromatin structure can be active or inactive, depending on the packaging of proteins and modification (such as methylation) of DNA (Hinnebusch et al 1994). The transcription process can be regulated at initiation, elongation, degradation, and termination. Additional controls can be

exerted in regulating transacting proteins, and even the DNA sequence itself may influence gene expression. Posttranscriptional regulation may also be important and includes exon excision and intron ligation and RNA editing (insertion, deletion or replacement of nucleotide as a way of regulation) (Lithgow et al 1997). mRNA transport from the nucleus to cytosol and stability of RNA are also two ways in which gene expression can be controlled. Translational control is another critical control point. It involves modification of the translational initiation machinery, regulation of availability of tRNA, and effects on ribosome assembly. Protein degradation and recycling are also important ways to regulate protein activity. All these potential sites of regulation are also related to the growth status of cells, including extracellular environmental changes (such as nutrients), stress, and the stage of the cell cycle. These signals are mediated by numerous cell receptors and a variety of protein kinases. All these events are further complicated by restriction of events in organelles, such as nuclei or mitochondria. Furthermore, cross-talk between these regulatory pathways present an even more complex picture. My focus in this study is on one major controlling event in this picture: transcriptional initiation particularly how the CCR4-NOT complex and ADR1 play their roles in regulating gene expression in yeast.

Access of transcriptional factors to the DNA may be regulated by chromatin structure. Chromatin remodeling may therefore be often required for transcription activation. Nucleosomes may be the most abundant repressors of gene transcription. Disassembling the nucleosomal packaging is an ATP-driven process and, in many cases, requires SWI/SNF or other DNA or nucleosome-stimulated ATPase activity (Ryan et al 1998). SWI/SNF consists of at least 11 subunits and its essential components include the DNA-dependent ATPase SWI2/SNF2. This activity increases the binding of certain activators and is required for chromatin remodeling and transcriptional activation (Natarajan et al 1999). Other complexes with ATP-dependent activity in yeast include RSC (remodel the structure of chromatin) and the ISWI group (Vignali et al 2000). SNF/SWI-dependent chromatin remodeling is required but not sufficient for transcription activation. However, the extent of nucleosome depletion in vivo is correlated with the degree of derepression (Han et al 1988). In addition, SNF/SWI interact with the SRB mediator associated with RNA polymerase II and another chromatin remodeling complex, SAGA, probably creates a synergistic action on nuclesome remodeling and transcription activation (Roberts et al 1997).

Modification and remodeling of chromatin also involves the SAGA (SPT-ADA-GCN5-Acetyltransferase) complex. This complex affects a number of genes such as those involved in amino acid metabolism, mating pheromone response, mating-type switching, and carbon source utilization (Robert et al 1997). The histone acetyl transferase (HAT) activity of GCN5 acetylates lysine 14 in histone 3 and lysine 8 and lysine 16 in histone 4 in vivo (Kuo et al 1996). While GCN5 alone can not acetylate whole nucleosomes, the SAGA complex is capable of effectively acetylating histones in nucleosomes (Ogryzko et al 1998). Hyperacetylation is associated with active chromosomal loci while hypoacetylation with inactive chromatin (Kuo et al 1998). Chromatin remodeling is targeted at the promoter but not the whole transcription unit (Imhof et al 1998). The access of the transcription factors can be increased by acetylation through topological change (Lee et al 1993, Wang et al 1998). SAGA has been shown to be the target of a number of activators (Grant et al 1998). It has also been found closely associated with the polymerase II holoenzyme and it shares some components with TFIID, which also has HAT activity in one of its component TAF130 (Grant et al 1998). Acetylation affects the transcription activity of the chromatin template and interactions of

histones with nonhistone proteins. In contrast, histone deacetylase is a target of a variety of repressors. Though each of the SAGA components affect only a subset of genes (Struhl 1998), the effects are based on analysis conducted on rich medium and no similar test has been conducted under the conditions of amino acid starvation. In addition to its minimal effect on 5% of total genes by GCN5 in yeast, SAGA might play a broader role under other conditions (Holstege et al 1998).

In addition to the chromatin remodeling systems that affect transcription, the assembly of the transcription machinery may also be rate-limiting for gene expression. RNA polymerases and their associated factors are the basic machinery for performing transcription. Gene transcription is performed by three classes of RNA polymerases. Polymerase I transcribes ribosomal RNA (rRNA), polymerase II message RNA (mRNA) and some Sn RNAs, and polymerase III transfer RNA (tRNA), 5S RNA and the remaining of Sn RNAs. All three classes of polymerase depend on TBP, the TATA-box binding protein for initiating transcription. Transcription by RNA polymerase II can be divided into basal and regulated transcription. Basal transcription requires TFIIB, TBP, TFIIA, TFIIE, TFIIF, TFIIH and RNA polymerase II. Regulated transcription recruits at least two complexes, TFIID (TBP) and RNA holoenzyme (O'Neill

et al 1995). Purified RNA holoenzyme consists of RNA polymerase II, general transcription factors (TFIIB, TFIIE, TFIIF, and TFIIH), SRBs and mediator components. Transcription activators recruit the TFIID, TFIIA, TFIIB, and/or RNA polymerase holoenzyme to initiate gene transcription.

Activated RNA polymerase II transcription requires the participation of TFIIB, TFIIE, TFIIF, TFIIH, and, in many cases, TFIIA, and TBP-associated factors. In addition, TBP is usually required. TFIID in yeast consists of TBP and 12 TBP associated factors (TAF_{II}s) (Table 2), while a number of distinct TFIID complexes associated with distinct set of genes may exist in higher organisms (Chicca et al 1998). Despite the different forms of TFIID, all mediate signal transduction to the transcription machinery. They function in promoter recognition, transcription activation or repression, histone acetylation and chromatin remodeling and cell cycle progression. TAF_I and TAF_{III} are TBP associated factors required for rRNA and tRNA, respectively. TAF proteins in this study all refer to the type II TAF proteins that affect the mRNA transcription unless indicated otherwise. Most TAF proteins are well conserved from yeast to human. In contrast to the SAGA complex, TFIID is capable of binding promoter DNA. Some studies have suggested that TBP binding to TATA element is rate-limiting for assembling an active RNA polymerase II holoenzyme (Lee et

al 1997, Li et al 1999, and Kuras et al 1999). However, there is evidence that TBP is constantly present on some transcriptionally inactive but inducible promoters (Gross et al 1990, Chen et al 1994). In this case, the conformational changes induced by transcription activators are suggested to be rate-limiting. Most recent results, however, showed that TFIID dimerization is rate-limiting for uninduced transcription at certain promoters (Jackson-Fisher et al 1999). Or, alternatively, the rate-limiting step occurs when TFIID is recruited to the promoter as a whole complex or component by component (Gonzalez-couto et al 1997). Therefore, several of the assembly processes could be rate-limiting for transcription initiation.

TFIIA is composed of 2 subunits of TOA1 and TOA2 and stimulates both basal and activated transcription at some promoters (Kang et al 1995, Ma et al 1996). It is suggested that it stabilizes the TBP-DNA complex (Ozer et al 1998). TOA1 competes with TAF130 N-terminal to bind TBP in vitro. In addition, overexpression of TFIIA can suppress the growth defect of a TAF130 N-terminal deletion (Kokubo et al 1998). Therefore, TFIIA may be a coactivator while TAF130 might participate in chromatin remodeling through its HAT activity (Kang et al 1995, Lieberman et al 1997). The role of TFIIA may be a result of counteracting

the repressing role of MOT1 that disrupts the TBP-TATA interaction (Madison et al 1997). The coactivator role of TFIIA has been further substantiated by the fact that transcriptional activators such as VP-16 and Zta bind to TFIIA and stimulate transcriptional activation (Kobayashi et al 1995).

TFIIB/SUA7 in yeast was isolated as a suppressor of a start site alteration in the cyc1 promoter (Pinto et al 1992). This is the same phenotype caused by a mutation in the largest subunit of RNA polymerase II, consistent with the known role for TFIIB in transcription start site selection (Pardee et al 1998). TFIIB is capable of interacting with TFIIF, RNA polymerase II, and connecting TFIID to the remaining of the RNA polymerase holoenzyme (Bangur et al 1997, Goodrich et al 1993). TFIIB binds simultaneously with TFIIA to the TBP-TATA complex and there is no overlapping contacts and no direct contact with each other (Keener et al 1997). TFIIB and TFIIA stabilize the orientation, but do not fix the orientation of TBP binding to the TATA element. The C-terminal domain of TFIIB interacts with TBP, though this interaction is not rate-limiting for most genes.

TFIID and TFIIA are suggested to start transcription initiation by binding to a promoter TATA-box upon activator binding. Then,

polymerase II is recruited to the promoter at least partially through the function of TFIIB and the carboxy terminal domain of the largest subunit of RNA polymerase II which in this active preinitiation complex (PIC) is not phosphorylated (Ranish et al 1999). The recruited TFIIB assists the recruitment of TFIIF and RNA polymerase II by direct interaction with TFIIF. TFIIF consists of two subunit, RAP74 and RAP30, and both are believed to participate in the recruitment of RNA polymerase II (Henry et al 1994).

TFIIE and TFIIH are subsequently assembled into this RNA polymerase PIC formation. Upon ATP hydrolysis, an open complex is formed. The change of a closed inactive PIC to an open active PIC requires ATP, TFIIH helicase activity and TFIIE, which has been suggested to regulate the ATPase, helicase, and kinase activity of TFIIH (Henry et al 1994). However, recent studies also showed that TFIIE may participate in TFIID funtion and TFIIH contributes to promoter melting (Yokomori et al 1998). The helicase and ATPase activities of TFIIH are suggested to participate in the last rate-limiting steps before initiation (Yan et al 1997, Holstege et al 1997). In addition to its role in promoter escape, TFIIH also participates in nucleotide excision repair (NER) and possibly in repairing actively transcribed genes (Svejstrup et al 1996).

TFIIH is also one of the three kinases that phosphorylate the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II. So far at least three CTD kinases have been identified, each of which pair up with a cyclin to form a regulated kinase. They are CTK1/CTK2, KIN28/CCL1 (KIN28 is the catalytic subunit of TFIIH), and SRB10/SRB11 (Andrews et al 1998, Kuchin et al 1998). These three kinases, KIN28, CTK1, SRB10, are suggested to phosphorylate the CTD at different residues at distinct times (Hengartner et al 1998). For instance, SRB10 phosphorylates CTD at Ser 2 and Ser 5 whereas TFIIH at Ser 5 only (Trigon et al 1998). Functionally, CTD phosphorylation by SRB10 is inhibitory, occurring in a preinitiation process, whereas TFIIH phosphorylation of CTD stimulates transcription when occuring later in the transcription initiation process (Hengartner et al 1998).

Activated transcription requires activator binding to DNA, which induces chromatin remodeling and transcription initiation. Activators can interfere with or disassemble the repressing chromatin or increase the affinity of GTF for chromatin (Wilson et al 1996). In addition, activators often interact with multiple components in the RNA polymerase II holoenzyme directly or indirectly to initiate transcription. The interactions of activators with RNA polymerasse II holoenzyme are usually mediated by

adaptors/mediators /cofactors, which include SRBs (suppressor of RNA polymerase B, which is later named RNA polymerase II) and TAFs. Mediator activity was originally identified by a squelching assay (Flanagan et al 1991). Addition of one activator could interfere with the stimulation of transcription by another, indicating that they share a common target. The mediator complex plays an intermediary role between activators or repressors and the CTD of RNA polymerase II (Kuchin et al 1995). Subsequent biochemical data revealed that mediator activity cofractionates with RNA polymerase II and with TFIIF when the antibody against the CTD of the largest subunit of RNA polymerase II was used (Kelleher et al 1990).

One of the purified mediator complexes contains about 20 polypeptides. It is capable of supporting and stimulating basal and activated transcription and also stimulates the phosphorylation of the CTD by TFIIH. Other evidence shows that the mediator complex is important for both transcription activation and repression in vivo (Sayer et al 1992). Many of these mediator proteins were identified as suppressors of a phenotype associated with truncation of the CTD of RNA polymerase B. These proteins were called SRB (Ushera et al 1992). In addition, a mammalian RNA polymerase II purified with the antibody

against one of the SRB also contains TFIIE and TFIIH, indicating close association of the mediator with the GTF (Chao et al 1996). The SRB complex appears, therefore, to connect transcription factors with the general transcriptional machinery. SRB mediator proteins (SRB2-6 and MEDs) are required for gene-specific activation, while SRB8-11 are suggested to be involved in transcription repression.

In addition to the trans-acting factors, cis DNA elements also affect RNA polymerase II transcription. Cis-acting elements include enhancers or upstream activating sequences (UAS, binding site of activators), silencers, and core promoters. The core promoter consists of the TATA box, transcription start site, and perhaps other sites where other factors bind (Mosch et al 1992, Lagrange et al 1998). The TATA-box is located about 40-120 nucleotide from the transcription start site in yeast but 30 nucleotides in higher eukaryotes (Hahn et al 1989). At many promoters there are extra cis-elements such as the initiator element (encompassing the transcription start site)(Mosch et al 1992) and the TFIIB recognition element (BRE) (Lagrange et al 1998). Core promoter strength is indicative of net total effects of these elements and their associated factors.

Recycling of RNA polymerase II and reinitiation are still under much investigation. The degree to which the promoter retains the TFIID-TFIIA complex after the release of the RNA polymerase II might be one of the determinants of reinitiation rate (Yean et al 1999).

Therefore, transcription regulation is controlled at multiple steps from initiation, elongation to termination and reinitiation and the whole process requires the coordinate action of numerous factors. My study is mainly focused on the interactions of activators with components involved in transcription initiation.

CHAPTER 1

BIOCHEMICAL INTERACTIONS WITH THE CCR4-NOT COMPLEX

Introduction

Yeast growth can be divided into fermentative and nonfermentative growth depending on the available carbon source. In general, yeast can grow on glucose (fermentative) or ethanol (non-fermentative). However, yeast prefer glucose as their carbon source and glucose represses expression of a variety of genes involved in carbon source metabolism other than glucose, for instance, galactose, sucrose, glycerol, and ethanol (Ronne et al 1995). Because yeast metabolism of glucose to ethanol can occur anaerobically, genes required for aerobic growth are switched off when cells are grown on glucose. This phenomenon is called glucose repression or carbon catabolite repression (Hardie et al 1994). Partially glucose-repressed cells exhibit a short cell cycle and a high rate of glycolysis. The ADHI enzyme is required for the metabolism of glucose to ethanol and its expression is reduced 10 times on ethanol (Denis et al 1983). The growth on ethanol or glycerol is called nonfermentative and requires the respiration chain and the citric acid cycle (Russell et al 1983,

Young et al 1985). The ADHII protein is required for the conversion of ethanol to acetaldehyde, which can be utilized in the citric acid cycle and glyoxylate pathways. ADHII is repressed by glucose and derepressed when glucose is depleted. The metabolic pathways involving ADHI and ADHII enzymes are summarized in Figure 1.

Multiple genes are involved in glucose repression and derepression. Glucose repression and derepression are closely related events. Proteins involved in glucose repression include, SSN20/SPT6, REG1, HXK1/2 and SNF1 (Ronne et al 1995). A REG1 mutation causes glucose-insensitive expression of galactose—related metabolism. HXK1/2 encode a hexokinase used in the glycotic pathway and its mutation reduces glucose repression and up-regulates REG1 expression (Ulery et al 1994). REG1 and SNF1 also affect derepression of mitochondria transcripts (Celenza et al 1989). SNF1/CCR1/CAT1 is a glucose-repressible serine/threonine protein kinase conserved from yeast to mammals (Hardie et al 1994). It was isolated as a mutant which can not grow on sucrose. In addition to affecting glucose-repressible genes such as SUC2, and COX6, it is unable to accumulate glycogen (Thompson-Jaeger et al 1991). The kinase activity of SNF1 is regulated by the regulatory subunit

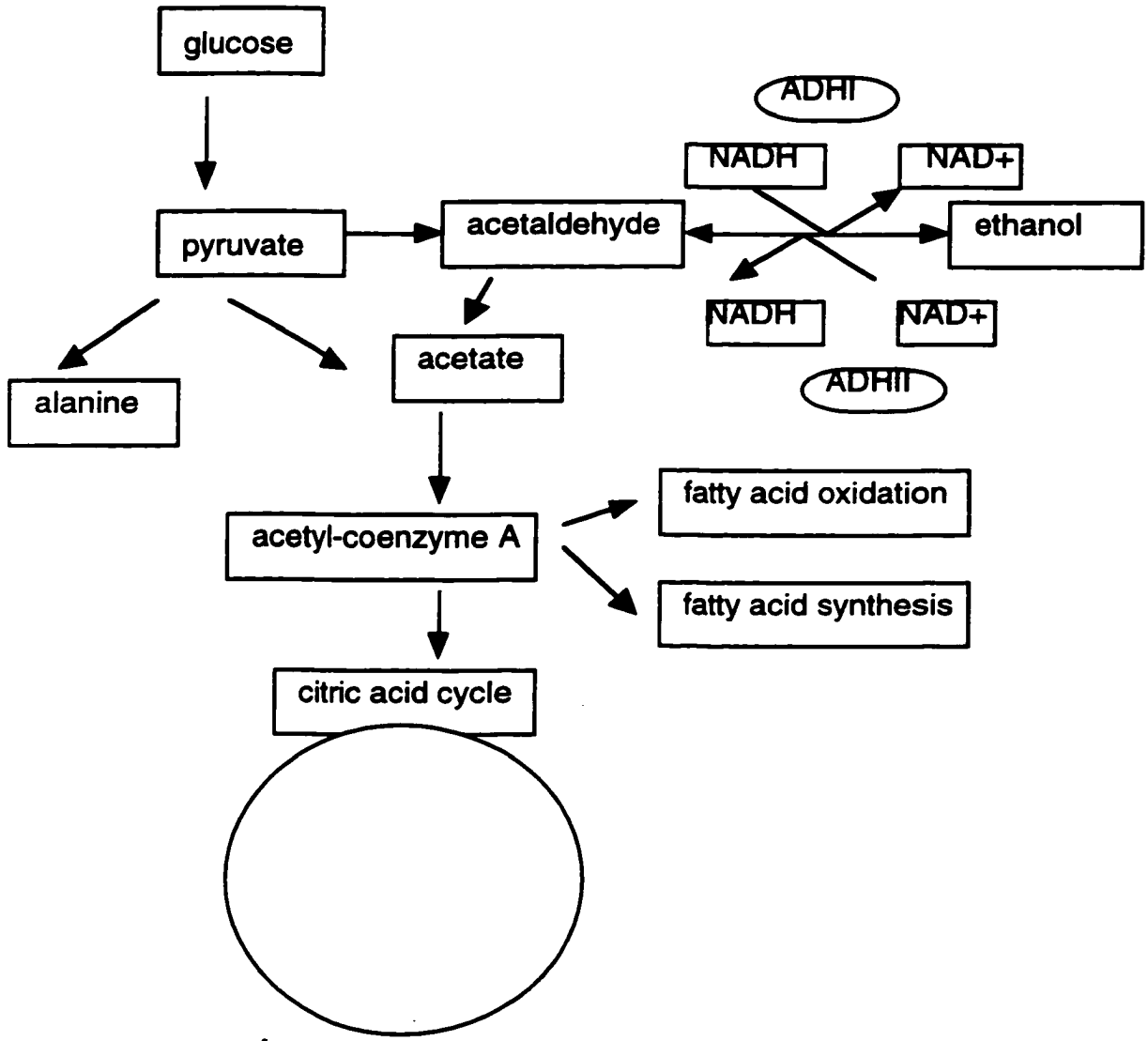

Figure 1. ADH enzymes in metabolic pathways.

SNF4 (Celenza et al 1989). SNF1-SNF4 can phosphorylate and inactivate enzymes involved in nonfermentative growth in vitro (Hunter et al 1997, Mitchelhill et al 1994). Association with SNF4 is regulated by glucose (Lee et al 1998, Lesage et al 1996) and SNF1 kinase activity is increased when glucose is depleted from the medium. The AMP:ATP ratio may regulate glucose derepression through regulating SNF1 kinase activity (Jiang et al 1997).

CCR4 and CAF1 are required for glucose derepression and ADH2 expression. Both of them have been purified with several NOT proteins and these proteins are characterized as a complex named CCR4-NOT (Liu et al 1998). The core of CCR4-NOT is a $1X10^6$ dalton (mD) complex (Liu et al 1998, Bai et al 1999). The 1 mD complex consists of CCR4, CAF1, NOT1-5 and three other uncharacterized factors (Draper et al 1994, 1995, Liu et al 1998, Y.C. Chiang and J. Chen, unpub. Observ.). A 1.9 mD complex was also identified and it apparently includes the core CCR4-NOT components. In addition, it may also includes DHH1 (Hata et al 1998), CAF4, CAF16 (Liu et al unpublished observation), DBF2 (Liu et al 1997), and MOB1 (Komarnistsky et al 1998). Importantly, the association of CCR4 with the 1.9 mD complex is not affected by a caf1

deletion whereas CAF1 is absolutely required for CCR4 association in the 1 mD complex (Liu et al 1998, Bai et al 1999).

CCR4 (837 amino acids (aa) in length) was originally identified by mutations that suppressed the increase of ADH2 mRNA levels caused by cre1/ spt10 (Denis 1984). CCR4 has five leucine repeats between residues 350-467 aa (Matvar et al 1990). The leucine-rich repeat is required for CCR4 association in the 1 mD CCR4-NOT complex (Draper et al 1994, 1995). CCR4 shares similar phenotypes with CAF1/POP2. Both affect ADH2 under glucose-derepressing conditions and they are capable of suppressing spt10-enhanced ADHII activity (Denis et al 1984, Draper et al 1995). They also display staurosporine and caffeine sensitivity (Hata et al 1998), temperature sensitive growth at 37°C on non-fermentative medium and cold-sensitivity (Sakai et al 1992, Liu et al 1997 and Hata et al 1998). CAF1 (444 aa in length) also contains some motifs typical of transcription factors such as a glutamine-rich region, proline-rich region and serine/threonine-rich region. It is strongly associated with CCR4 through its C-terminus (Draper et al 1995).

CCR4 and CAF1 mutations can suppress spt6 and spt10 phenotypes at the his4-912delta locus. his4-912delta contains a Ty insertion at the HIS4 promoter turning off normal HIS4 expression. SPT6/CRE2/SSN20

and SPT10/CRE1/SUD1 are proteins involved in several cellular process. SPT represents SuPpressor of Ty. spt6 and spt10 mutations increase ADH2 expression under glucose-repressing conditions and also alter HIS4 transcription at the his4-912 delta locus (Denis 1984, Clark-Adams et al 1987). SPT6 overproduction affects ADH2 expression both through its upstream activationg sequence 2 (UAS2) and other sequences (Donoviel et al 1996). Phenotypes of spt6 are similar to those caused by alteration in histone abundance, suggesting an SPT6 involvement in chromatin structure (Neigeborn et al 1987). In addition, SPT6 has been shown to interact strongly with histone H3 and weakly with histone H4 (Bortvin et al 1996).

CAF1 was also found to interact with SRB9/CAF3. SRB8-11 were originally isolated as suppressors of mutations in the protein kinase SNF1 which play a major role in glucose repression (Li et al 1995). SRB10/SRB11 is a cyclin-dependent kinase with a role in transcriptional repression by SSN6/TUP1, a global corepressor (Kuchin et al 1998). So far three CCR4 associated factors, including CAF1 in the core complex, have been found to interact with SRB9-11, suggesting that the CCR4-NOT complex may have close physical interactions with SRB9-11 proteins.

NOT proteins have been found to be part of the CCR4 complex and exhibit multiple phenotypes (Liu et al 1998). NOT proteins have been extensively studied at the yeast HIS3 promoter. The upstream of this promoter has two TATA elements, T_R is located at –47 to –40 and mediates transcription starting at +1 while Tc is located at –54 to –83 and starts transcription at +13 (Struhl 1986, Collart et al 1996). Only T_R can be activated by the transcriptional activator GCN4 (Colgan et al 1992, Ham et al 1994). NOT1/CDC39, NOT2/CDC36, NOT3, NOT4/SIG1/MOT2, NOT5 specifically repress Tc-dependent transcription (Collart et al 1993, 1994, Oberholzer et al 1998). Mutants in these genes cause cell resistance to 3-aminotriazol (3-AT), which is a competitive inhibitor of the HIS3 protein (Collart et al 1993). Tc is a non-typical TATA-less element, though it also requires TBP for transcription initiation (Cormack 1992). Compared with a canonical TATA element, transcription from a Tc promoter is much less efficient (Colgan et al 1992, Collart et al 1996, Ponticell et al 1990). The transcription from a TATA-less promoter is also less dependent on the TBP protein and the Tc sequence is resistant to point mutations (Iyer et al 1995). In addition, the Tc sequence does not yield DNA I footprints with TFIID nor does it show

micrococcal nuclease resistance (Collart et al 1993, Oettinger et al 1985).

NOT1 is an essential nuclear protein with two molecular forms, 185 kD and 195 kD in size. The 185 kD species is the more abundant form, lacking approximately 100 residues from the N-terminus (Liu et al 1998). The C-terminal 1490-2108 residues of NOT1 is required for cells to remain viable. Mutations in NOT1, NOT2(CDC36), NOT4 or NOT5 possess some unique features as compared to CCR4 and CAF1. For instance, the five NOT genes when mutated display 3-AT sensitivity in a gcn4 background. In contrast, ccr4 does not display this phenotype and caf1 exhibits only a weak 3-AT phenotype (Liu et al 1998). At the same time, phenotypes associated with loss of function of the NOTs share many similarities with the phenotypes associated with defects in CCR4 and CAF1 (Liu et al 1998), including caffeine sensitivity, temperature sensitivity, and increased sensitivity to magnesium. Taken together, while the NOT proteins and CCR4/CAF1 may function differently at some promoters, they probably share functions at other promoters.

NOT proteins may play their roles through interacting with TFIID. The repression at Tc by NOT proteins does not involve TUP1 or CYC8 (Collart et al 1993) nor does it occur through the inhibition of

transcription factors. not mutations can suppress TBP overproduction toxicity in mot-1 mutants (Collart et al 1996), indicating that there exist some genetic interactions between NOT proteins and TBP and its associated factor MOT1. NOT1 protein also immunoprecipitate with TBP, suggesting a physical interaction between NOTs and TFIID (Lee et al 1998). In addition, NOT2 and NOT5 have also been shown to coimmunoprecipitate with TBP and NOT5 also interacts with several TAF proteins (Badarinarayana et al 2000). Genetic interactions between CCR4-NOT components and TFIID were also characterized. In addition, NOT2 has also been shown to interact with components in SAGA, a complex required for chromatin remodeling and for transcription initiation (Benson et al 1998).

My research on CCR4-NOT is aimed at understanding the protein interactions within the CCR4-NOT complex and the physical positions of each component. Previously it has been shown that CCR4 binds CAF1 which in turn binds NOT1 (Liu et al 1998, Draper et al 1994) and that CCR4 and NOT proteins were identified through distinct genetic screens. Therefore, the hypothesis that CCR4 and CAF1 may associate independently of NOT proteins physically and functionally is tested using a series of gel filtration and immunoprecipitation experiments.

Material And Methods

Yeast strains growth conditions and enzyme assays

Yeast strains are listed in Table 1. Yeast were grown at 30 °C on YEP medium (2% yeast extract, 1% bactopeptone) or selective medium (Cook et al 1994) that was supplemented with 5% glucose or 2% galactose/raffinose. The yeast transformation protocol was described as previously (Ito 1983 and Cook et al 1994)).

DNA constructions

In order to purify large amount of proteins to produce antibody against DHH1, the yeast vector containing GAL-DHH1 was cut with Sma I and Xho I and ligated into a E. coli expression vector pEG-KG cut with the same two enzymes. This resultant GST-DHH1 construct was cut with EcoR I and the large fragment was filled in and self-ligated to give the GST-DHH1 N-terminus (1-266 aa) expression vector. The small fragment was ligated to pGEX-1 lamda cut with Sma I to yield the GST-DHH1 C-terminus (267-506 aa) expression vector.

Antibodies and immunoprecipitation

For western analysis the antibodies were directed against GST-CAF1, GST-NOT2, 6-His- NOT5, GST-NOT1 (1480-2109 aa), and GST-DHH1

Table 1. Strains and their genotypes

Strains	genotypes
KY803	MATa HIS leu2-PET56 trp1-Δ1 ura3-52 gal2 gcn4-Δ1
MY8	isogenic to KY803 except not1-2
MY1737	MATa his3::TRP1 leu2 trp1 ura3 not1::LEU2 pRS316-NOT1-396-2109
MY1738	MATα leu2 ura3 trp1 not1::LEU2 pRS316-NOT1-1319-2109
MY16	isogenic to KY803 except not2-1
1393-4a	MATα his3/his2 leu2 ura3 not2::TRP1
MY1735	Isogenic to KY803 except not5::URA3
MY508	isogenic to KY803 except not3::URA3
MY537	isogenic to KY803 except not4::URA3
EGY188	MATa adh1-11 ura3 his3 trp1 LexA-LEU2

(267-506 aa) fusion proteins. GST protein was purified as described (Frangion et al 1993). Western analysis was conducted as described (Draper et al 1995). Immunoprecipitations were carried out as previously described (Cook et al 1994, Draper et al 1995). The CAF1 antibody was partially purified as described (Hagop et al 1998).

Gel filtration chromatography

A Superose 6 10/30 column was first balanced with 10 volumes of running buffer(50mM Tris Oac, pH7.9, 150mMKOAc, 0.02% Tween-20). The protein extract was made with 3A extract buffer (150mM TrisOAc,pH7.9, 450mM KOAc, 30% glycerol, 5mM DTT, 3mM EDTA). The procedure has been described in detail (Liu et al 1998). The flow rate was 0.2 ml/min and 0.5 ml was collected per fraction. The precleared protein sample was loaded into a 200 ul loop and a fraction collector was then started to collect fraction after 6 ml of running buffer had passed through the column. Fractions 2 to 20 were analyzed by Western blot analysis. Molecular weights for each fraction was calculated based on the elution volumes of Blue dextran (7.5 ml), Thyroglobin (12 ml) and BSA (16 ml).

Results

Formation of the 1 mD CCR4-NOT complex does not require the presence of NOT3, NOT4 or NOT5 proteins.

It has been shown that the CCR4-NOT1 complex forms two multicomponent complexes with approximate molecular weights of 1.9 mD and 1 mD, respectively (Liu et al 1998). To determine whether a not5 deletion affects the association of CCR4 and CAF1 in these two complexes, the CCR4-NOT complexes from a not5 strain was analyzed by gel filtration analysis. To conduct this experiment protein extract from a not5 strain was analysed using a Superose 6 10/30 column (Figure 2). Both CCR4 and CAF1 copurified in 1.9 (fraction 3) and 1.0 mD (fraction 11-12) complexes in a not5 background (Figure 2, lower panel). Similarly, not4 did not have a significant impact on the formation of these two complexes (Figure 2). A not3 deletion, however, appears to have an effect on the complex as it caused CCR4 association to become less concentrated in the 1 mD complex and appeared to affect the stablity of the 1 mD complex. In conclusion, deletion of NOT3, NOT4 or NOT5 does not affect the stable association of CCR4 with CAF1 in either of these two complexes. This data agrees with other data indicating that within

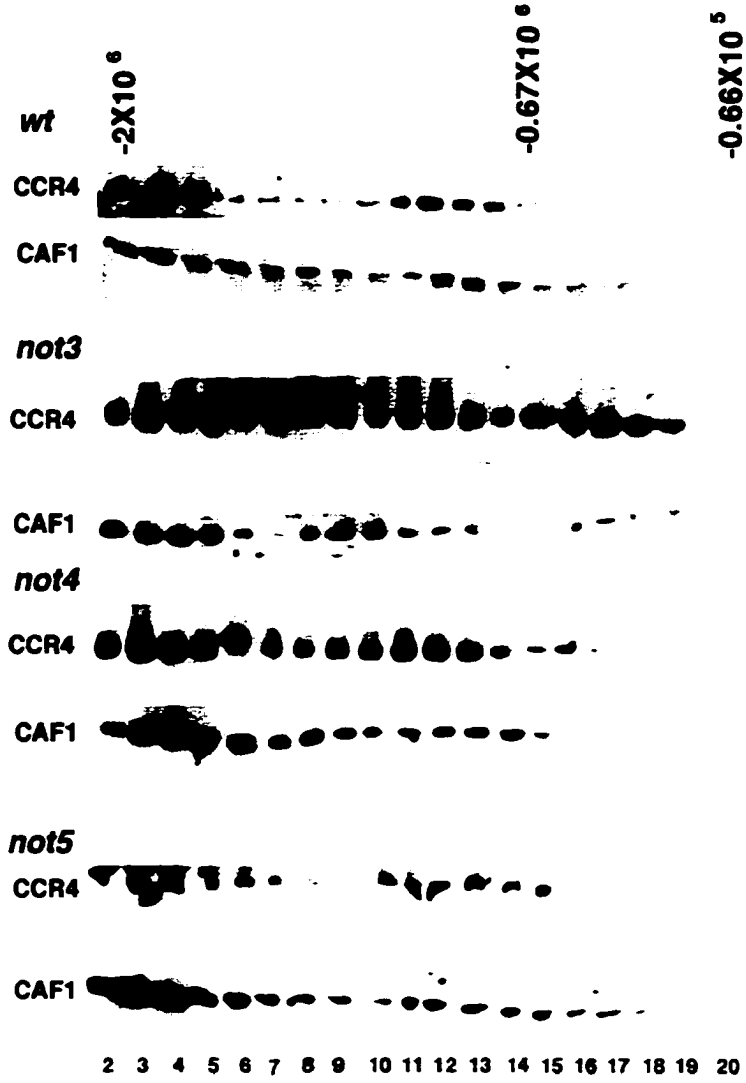

Figure 2. CCR4 associates with CAF1 in the absence of NOT3, NOT4, and NOT5. Yeast extracts from KY803(wt), 1393-4a (not2), and MY1735 (not5) were analyzed by gel filtration chromatography using a Superose 6 10/30 column. The protein extracts were precleared by centrifuging at 100,000g for 1 min. and 200 ul of sample was loaded onto the column. The flow rate was 0.2 ml/min and 0.5 ml volume was collected in each fraction. 100 ul from each fraction were analyzed by SDS-PAGE and Western blotting using CCR4 and CAF1 antibodies. The molecular weight markers for the gel filtration experiment were blue dextran ($2x10^6$ dalton), thyroglobulin ($0.67x10^6$ dalton), and BSA ($6.6x10^4$ dalton).

the CCR4-NOT complex that NOT3, NOT4, and NOT5 are physically separated from CCR4 and CAF1.

not2 affects association of CCR4 and CAF1 within the 1 mD complex.

Using a similar analysis as described above, the effect of not2 alleles on CCR4 and CAF1 association in the CCR4-NOT complexes was evaluated. As shown in Figure 5A, CCR4 and CAF1 cofractionated at both 1.9 mD and 1 mD in wild-type KY803 strain. In contrast, CCR4 ran in a altered pattern in the not2 background (Figure 3). The not2-1 mutation reduced the CCR4 protein levels in the 1 mD complex while not2 deletion in a different strain background caused partial dissociation of CCR4 from the 1 mD CCR4-NOT complex. However, no conclusion in regard to CAF1 can be drawn based on the available data despite results for CAF1 were shown. Therefore, NOT2 at least partially contribute to the stable formation of the 1 mD CCR4-NOT complex.

The N-terminal 395 aa of NOT1 protein contribute to the association of CCR4 with CAF1.

After examining the effects of the NOT3, NOT4, NOT5, and NOT2 components on the association of CCR4 and CAF1 in the 1 mD complex, the effect of another major component NOT1 was also investigated using different NOT1 deletion or mutation strains. First, the association of CCR4

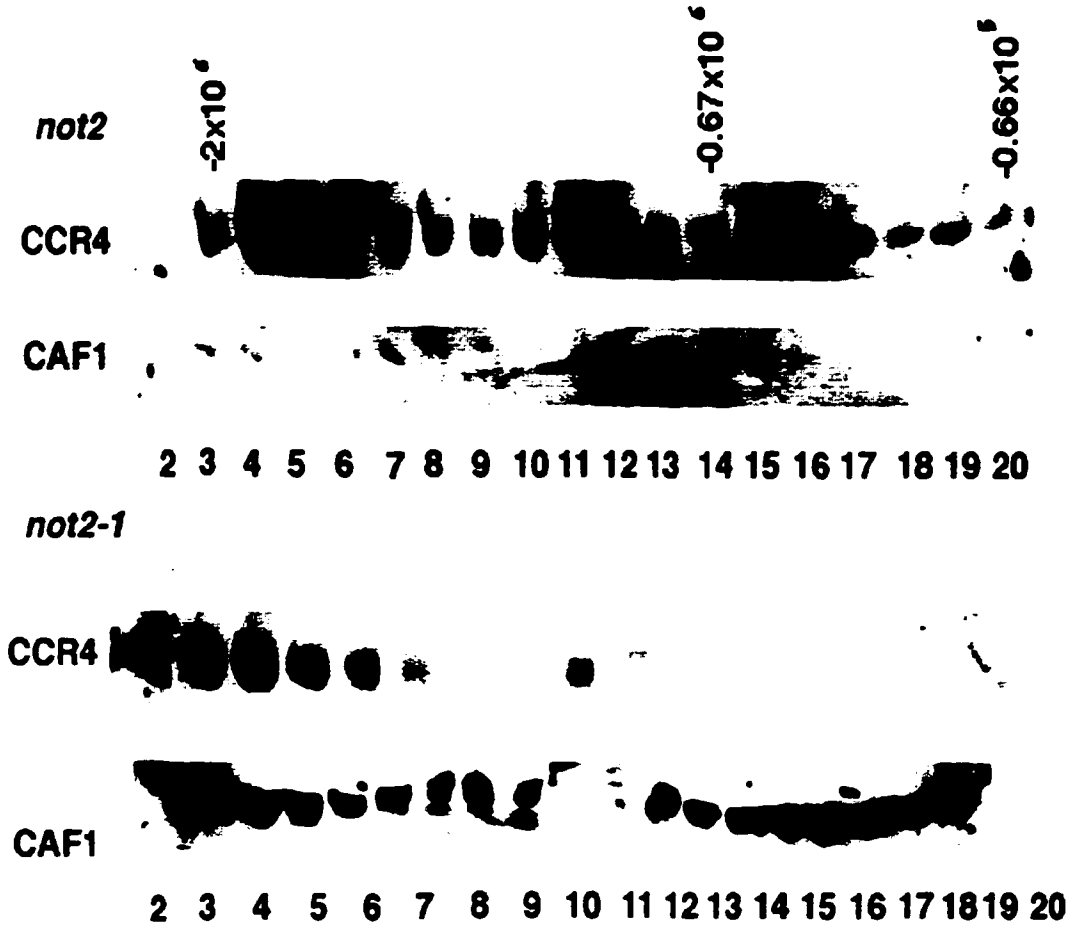

Figure 3. Gel filtration of extracts from not2 deletion and mutation strains. Strains MY16 (not2-1 mutation) and 1393-4a (not2 deletion) were grown and analysed as described in figure legend 2.

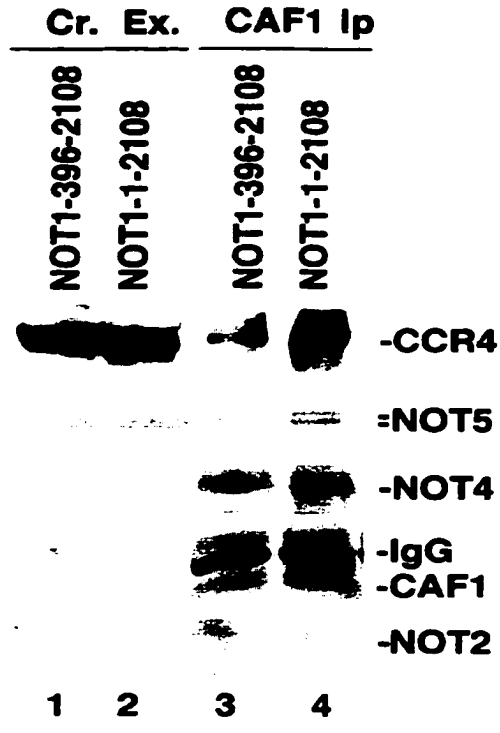

Figure 4. The N-terminal 396 residues of NOT1 is not required for the association of CCR4-NOT complex. Immunoprecipitations were conducted in strain MY1737 (not1 pNOT1-396-2108) and wild-type backgrounds with CAF1 antibody. Lanes 1 and 2 contain one tenth of the crude extract protein input used for the immunoprecipitations in lanes 3 and 4. Western analysis was conducted using anti-CCR4 and -NOT antibodies as indicated. NOT4 and NOT2 proteins in the crude extracts in lanes 1 and 2 were visible in the original westerns and were in equal abundance for the two strains. An ECL-based system was used for CCR4 westerns for lanes 1-4, whereas an alkaline phosphatase-based system was used for the remainder of the results. Molecular weights are indicated at the left.

with CAF1 was examined in a N-terminal deletion strain of NOT1. As shown in Figure 4, CAF1 immunoprecipitated the CCR4 protein as well as NOT4, NOT5 and NOT2 proteins in not1-396-2108 and wild-type strains. Compared with the wild-type, the CCR4 protein level brought down with CAF1 antibody in not1-396-2108 background is reduced while other proteins were not affected significantly. Therefore, NOT1 residues 1-395 are required for the complete association of CCR4 with CAF1.

The N-terminal half of NOT1 was required for forming the 1 mD CCR4-NOT complex.

Three not1 mutation or deletion strains were analyzed by gel filtration chromatography. First, a not1-2 strain expressing about 90% of truncated N-terminal NOT1 that is about 1100 aa in length and 10% of full-length NOT1 was analyzed (Bai et al 1999, Figure 5D). Though the not1-2 strain shared some similar phenotypes as not1-1319-2108, such as temperature sensitivity and 3-AT resistance, the CCR4-NOT complex in a not1-2 background was distinct from that observed in a not1-1319-2108 background. The gel filtration profile of not1-2 showed that CCR4 and CAF1 were still associated in the 1.9 mD complex, but there was a slight shift of the 1 mD peak for about 2 fractions, equal to a MW shift of about 200 kD. Second, the NOT1 N-terminal deletion strain was

examined (Figure 5C). not1-396-2108 had no effect on CCR4 association in either the 1.9 or 1 mD complexes, although it did affect CAF1 association in the 1 mD complex. The fact that in a not1-396-2108 background CCR4 was still present in the 1.9 mD and 1 mD complexes and that CCR4 could immunoprecipitate with CAF1 antibody (Figure 4) may explain why a not1-396-2108 strain is not temperature sensitive and has no effects on ADH2 expression.

Third, the effect of deleting the N-terminal 1318 aa of NOT1 was analyzed using gel filtration (Figure 5B). The not1-1319-2108 strain is temperature sensitive and has decreased ADHII activity (Bai et al 1999). Removing residues 1-1318 of NOT1 caused the dissociation of CCR4 and CAF1 from the 1 mD complex, suggesting that amino acids 1-1318 of NOT1 is required for the physical integrity of this CCR4-NOT complex. Also, in a not1-1319-2108 background very little CAF1 was observed following gel filtration chromatography. Deletion of the N-terminal domain of NOT1 might cause severe instability of CAF1 in the CCR4-NOT complex and result in dissociation of most of the CAF1 from both the 1.9 mD and 1 mD complexes.

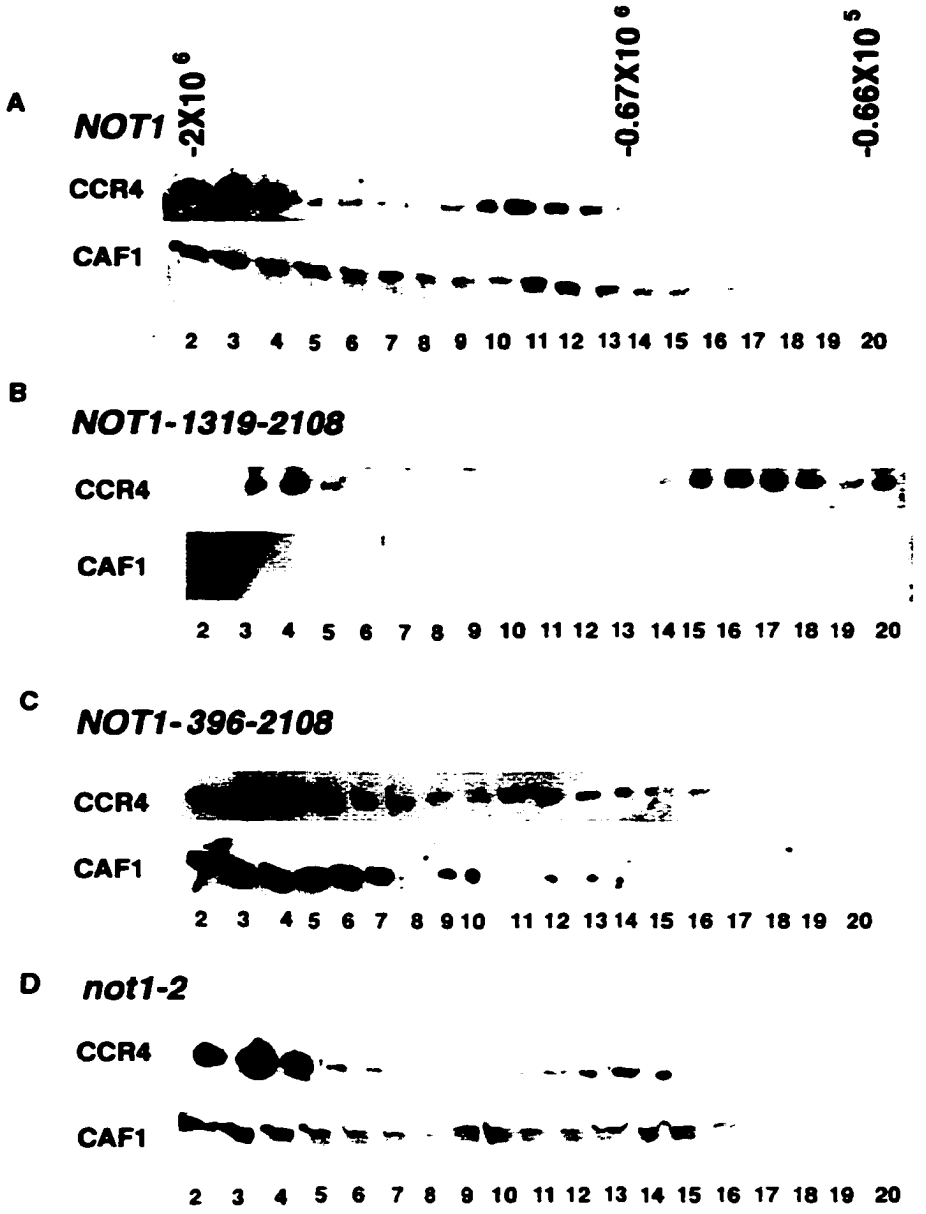

Figure 5. Gel filtration analysis of CCR4 and CAF1 in NOT1 mutant backgrounds. A- strain KY803 (wt); B- strain MY1738 (not1 pNOT1-1319-2108); C- strain MY1737 (not1 pNOT1-396-2108); D- strain MY8 (not1-2). Gel filtration chromatography was conducted as described in Figure 3. Anti-CCR4 antibody was used to detect the CCR4 protein.

Effects of not mutation on CCR4, CAF1, and NOT5 protein levels.

In order to determine whether the running pattern of CAF1 protein in gel filtration analysis is due to the decreased CAF1 in the crude extract, a series of western analysis were conducted in various not mutant backgrounds (Figure 6A). DHH1 protein levels was used as an internal control because DHH1 is not a component of the 1 mD CCR4-NOT complex. As shown in Figure 6A, CAF1 protein levels were significantly lowered in not1-1319-2108 and not2 backgrounds and NOT5 levels were decreased significantly in the not2 and not1-1319-2109 backgrounds. However, this reduction in CAF1 protein level can not alone explain the extreme low level of total CAF1 in all fractions of not1-1319-2108. CAF1 protein may also become more susceptible to protease degradation when N-terminal 1318 aa of NOT1 is deleted.

Carbon source and the natural genetic background are two factors that contribute to the difference in CCR4 and CAF1 levels in wild-type strains. The CCR4 level in the wild-type strain such as EGY188 is severely repressed under glucose growth conditions compared with that on galactose or ethanol medium (Figure 6B). When the sequence surrounding CCR4 is changed in strains like MD 9-7c(+) (MAT* adh1-11 his3 trp1 ura3

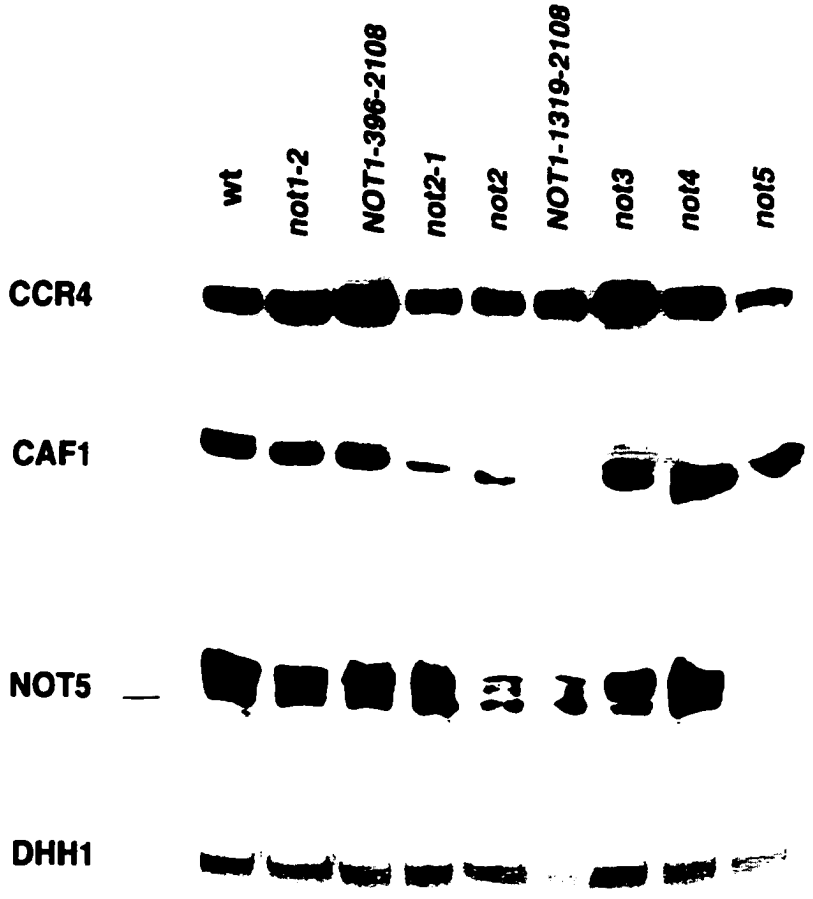

Figure 6A. CCR4, CAF1, and NOT5 protein levels in not mutant backgrounds. All strains were grown to mid-log phase in YEP medium containing 5% glucose. Cells were harvested, broken with glass beads, and 40 ug of total protein was loaded in each lane. The RNA helicase homolog DHH1 was used as an internal control to demonstrate equivalent loading onto the SDS-PAGE gel. Strains used are KY803 (wt), MY8 (not1-2), MY1737 (not1 pNOT1-396-2108), MY1738 (not1 pNOT1-1319-2108), MY16 (not2-1), 1393-4a (not2), MY508 (not3), MY537 (not4), MY1735 (not5). Western blot analysis was conducted with antibodies directed against CCR4, CAF1, NOT5, and DHH1 as indicated.

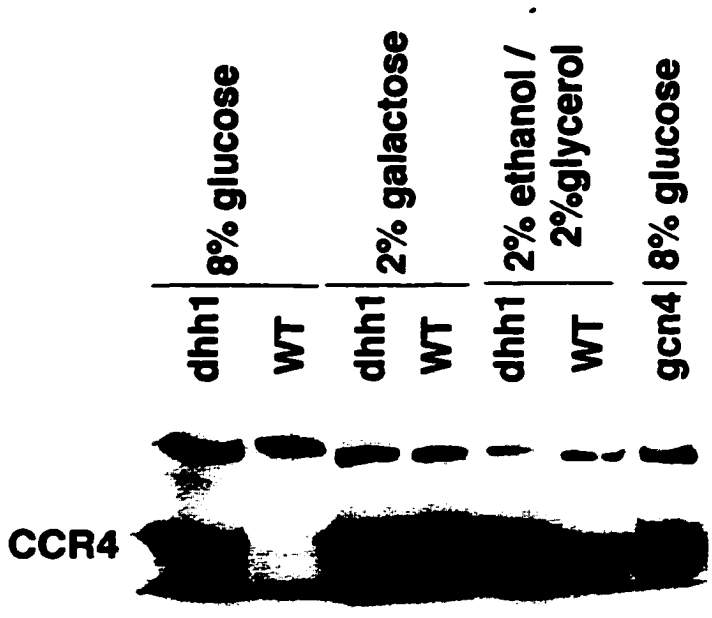

Figure 6B. CCR4 levels in wide-type, dhh1, and KY803 (gcn4) strains. Yeast were grown on 5% glucose, collected and extracts were prepared fresh without prior freezing. Proteins were measured with Bradford assay. 40 ug of protein extract was loaded for each lane and western blot analysis was conducted with antibodies against CCR4.

ccr4-10::CCR4-TRP1), the difference due to carbon source no longer exists (Draper et al 1994).

Comparing the CCR4 and CAF1 levels from the KY803 (gal2 gcn4) strain with that of the GAL2 GCN4 strain EGY188, the CCR4 level is dramatically increased in a gcn4 strain on glucose medium. This suggests that there might be a cross interaction between the pathways of amino acid synthesis and glucose repression or low levels of amino acids are associated with gluocose derepression.

Conclusions and Discussion

The N-terminal domain of NOT1 is required for the formation of the CCR4-NOT 1mD complex.

There are at least three lines of evidence that support the N-terminal domain of NOT1 as being required for formation of the functional CCR4-NOT complex. First, the N-terminal 1-1152 residues of NOT1 can be immunoprecipitated using either CCR4 or CAF1 antibody (Bai et al 1999). Moreover, the 667-1152 residue piece of NOT1 is sufficient for association with CCR4. Second, deletion of the N-terminal domain of NOT1 reduces the ability of ADH2 to derepress to the same extent as a

not1 mutation. ADH2 expression appears closely related to the formation of a functional CCR4-NOT complex. Third, gel filtration chromatography using strain not1-1319-2108 showed that without the N-terminus (1-1318 residues) of NOT1, both CCR4 and CAF1 dissociated from the 1mD complex and probably exist as free individual protein molecules or subcomplexes composed of at most several proteins.

NOT2 plays an important role for the 1 mD CCR4-NOT complex.

Functionally, not2 decreases ADH2 derepression by 8 fold (Liu et al 1998), similar to the effects of ccr4 or caf1. In contrast, not3 and not4 strains decrease the ADH2 expression by at most two-fold, and deletion of either of them did not apparently affect the CCR4-NOT complex. Therefore, there is a strong correlation between physical integrity of the complex and functional activity in terms of ADH2 expression.

The integrity of the 1 mD complex is correlated with CCR4-NOT funtion.

The 1mD complex, compared with the 1.9 mD complex, is an indicator of the functional CCR4-NOT complex. The 1.9 mD complex remains intact in both not1 and not2 mutation and/or deletion strains while the 1 mD complex is disrupted to different extents. In addition, genetic data also showed that the functions of CCR4-NOT were also severely affected when the 1 mD complex is disrupted (Bai et al 1999).

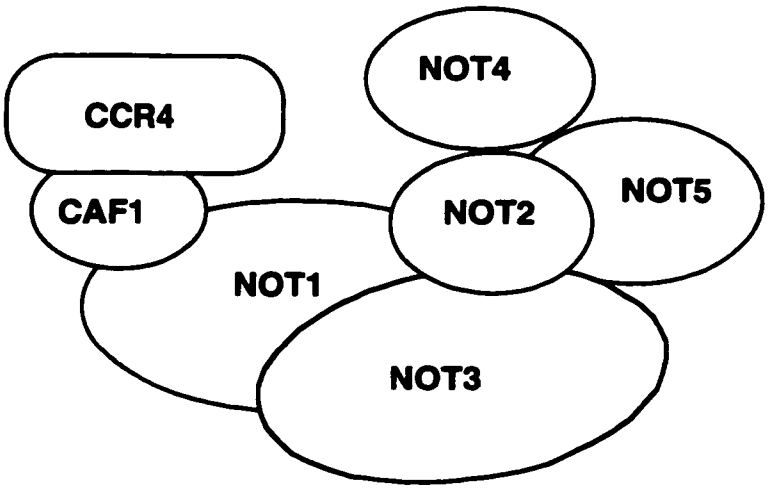

Figure 7. Model for protein contacts in CCR4-NOT complex. Based on the results presented herein CAF1 is presumed to bind to 667-1152 of NOT1, CCR4 binds to CAF1, and NOT2 and NOT5 interact with the C-terminal 1490-2108 residues of NOT1 in no particular order. NOT4 is placed on the periphery of NOT2 and NOT5 and it is presumed that NOT3 makes contacts to both NOT2, NOT5, or NOT4 and to the N-terminus of NOT1.

Combined with previous data (Bai et al 1999), a model is proposed concerning the physical association of the 1mD CCR4-NOT complex (Figure 7). Association of CCR4 with NOT1 is mediated by CAF1. CAF1 binds to the N-terminus domain of NOT1, while NOT2, NOT4 and NOT5 bind to the C-terminus domain of NOT1. NOT3 has an extensive but weak interaction with various components in this complex.

CHAPTER 2

INTERACTIONS OF THE TRANSCRIPTIONAL ACTIVATOR ADR1 WITH TFIID COMPONENTS

Introduction

Transcriptional activators turn on the expression of specific genes through interactions with transcriptional components such as TFIID, SRB proteins, and TFIIB (Bangur et al 1997, Lee et al 1998, Lagrange et al 1998,and Figure 8). Activators such as GCN4 and GAL4 have been extensively studied. GCN4 (280 aa in length) is required for genes induced by amino acid starvation (Hope et al 1986) and it has two functionally redundant activation domains each of which alone can support efficient activation of GCN4 targeted genes (Drysdale et al 1998). GCN4 has been shown to interact with the SWI/SNF complex (Neely et al 1999), SAGA components (Silverman et al 1997), SRB mediator subunits (Natarajan et al 1999) and TFIID components (Drysdale et al 1998). Similarly, GAL4 has also been shown to interact with SWI/SNF (Burns et al 1997), SRB10 (Hirst et al 1999) and TBP and TFIIB (Wu et al 1996). Another activator protein is the ADR1. It is

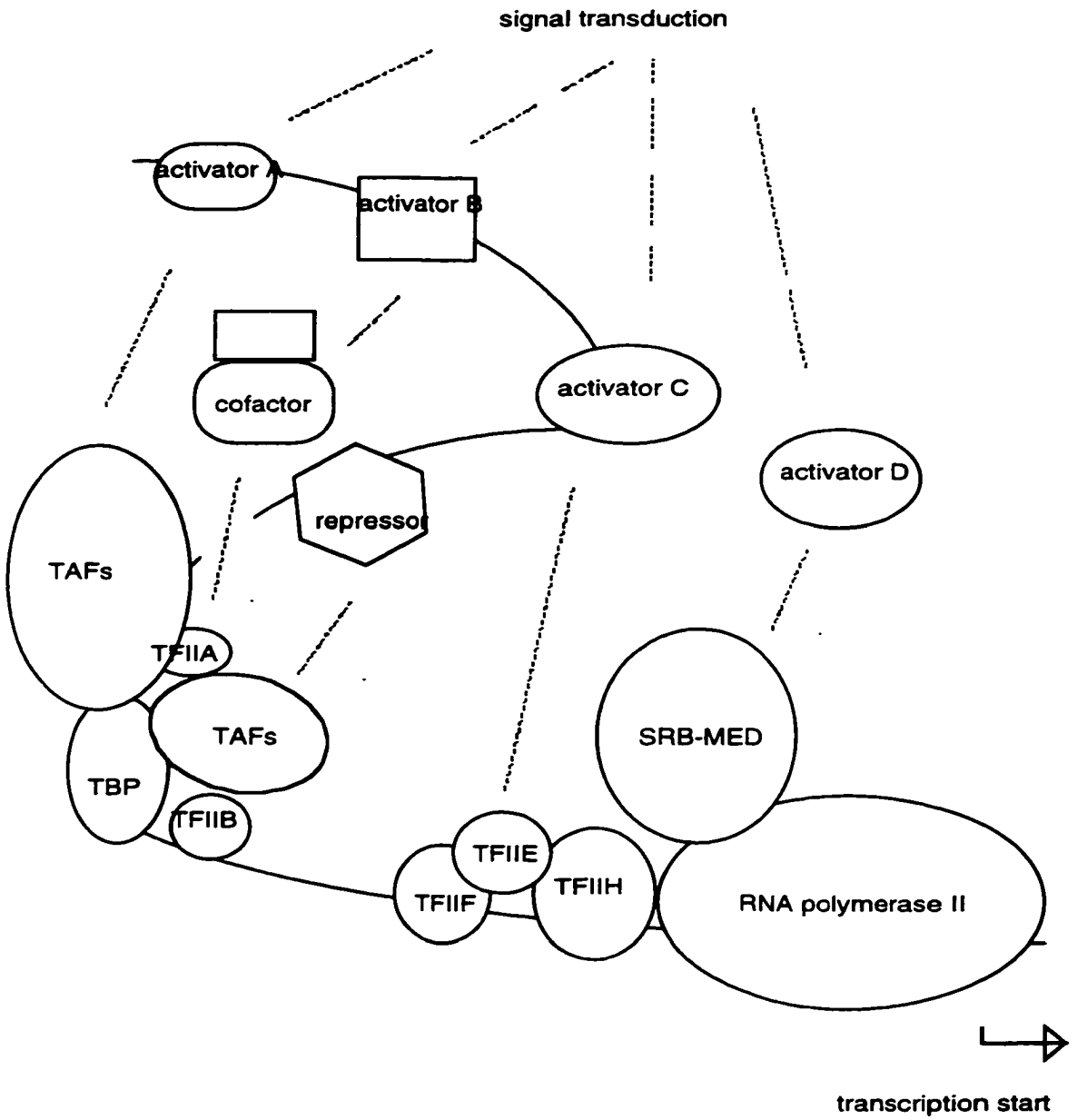

Figure 8. A proposed model on activated transcription. This model was based on the following references: Balciunas et al 1995 and Werner et al 1997.

essential for the nuclesome conformational change at the ADH2 gene (Verdone et al 1996, 1997) and interactions of ADR1 with the general transcription machinery is required for transcriptional activation of ADH2 gene (Chiang et al 1996, Komarnitsky et al 1998b).

The ADH2 gene has two UAS elements. UAS1 is a 22 bp palindrome sequence and is ADR1-dependent and UAS2 is SPT6- and MEU1-dependent (Multicopy Enhancer of UAS2, Donoviel et al 1996). Deletion of UAS1 causes a 10-fold reduction of ADH2 expression, but does not abolish ADH2 transcription. Deletion of UAS2 also severely reduces ADH2 expression and the UAS2-dependent ADH2 expression is repressed by glucose. Inactivation of MEU1 causes only 2-fold reduction in ADH2 expression, indicating the presence of other UAS2-dependent activator proteins (Donoviel et al 1996). The correct phasing between UAS1 and UAS2 (25 bp apart) is important and UAS1 and UAS2 appear to activate ADH2 expression synergistically. ADH2 expression is regulated by glucose repression and no induction is required. However, a repressor may be removed when ethanol or glycerol is the only available carbon source. The expression of ADH2 is increased several hundred-fold following growth on a nonfermentable carbon source (such as ethanol) as compared with growth on a fermentable carbon source (such as

glucose). Several repressors are known to control glucose repressed genes in yeast. In the case of ADH2, REG1 but not SSN6 or TUP1 is required for glucose repression of ADH2 expression (Dombek et al 1993). Inactivation of REG1 increases ADH2 transcription either directly or indirectly through affecting ADR1 expression and modification (Dombek et al 1993). Glucose regulates ADR1 mRNA stability and the rate of ADR1 protein synthesis but not ADR1 transcription rate (Vallari et al 1992). Post-translational modifications may also participate in ADR1 regulation (Cook et al 1994b).

ADR1, SNF1, and SNF4 proteins function as global regulators of glucose-derepressible genes and have been shown to activate peroxisomal genes, such as CTA1, FOX2 and FOX3 (Simon et al 1991, 1992). The observation that CTA1 expression depends more on the action of ADR1 while FOX3 relies more on the SNF1 protein suggests that ADR1 and SNF1 proteins do not display simple upstream and downstream relationships (Simon et al 1992, Navarro et al 1994). For instance, SNF1 may act on factors other than ADR1.

As a transcriptional activator of ADH2, ADR1 functions by increasing transcription initiation and possibly the formation of an active RNA polymerase II holoenzyme. ADR1 binds to the UAS1 of the ADH2 gene as

a dimer (Cook et al 1994a, Blumberg et al 1987, Kratzer et al 1997). The minimal DNA-binding domain of ADR1 consists of two Cys2-His2 zinc finger motifs (residues 99-155) and an additional 20 residues N-terminal to the zinc finger motif (Schmiedeskamp et al 1997). A conformational change in this DNA binding domain is induced upon binding the UAS1 sequence (Hyre et al 1998). Both zinc fingers are necessary for ADR1 activity (Blumberg et al 1987). However, ADR1 binding to DNA is required but not sufficient for transcriptional activation in vivo (Yu et al 1989).

ADR1 is composed of four transcriptional activation domains (TADs), TADI (residues 148-262), TADII (residues 263-359), TADIII (residues 420-462), and TADIV (residues 642-704) (Cook et al 1994a, Chiang et al 1996, Figure 9). ADR1 also contains an inhibitory region (residues 227-239) deletion of which enhances ADR1 function (Cook et al 1994b). Mutations within this region allows ADH2 chromatin remodeling and enhances ADH2 expression on glucose. ADH2 chromatin remodeling requires ADR1. However, chromatin remodeling upon activator binding itself is not sufficient for the transcriptional activation of ADH2 gene (Verdone et al 1996, 1997). Although it has been reported that a

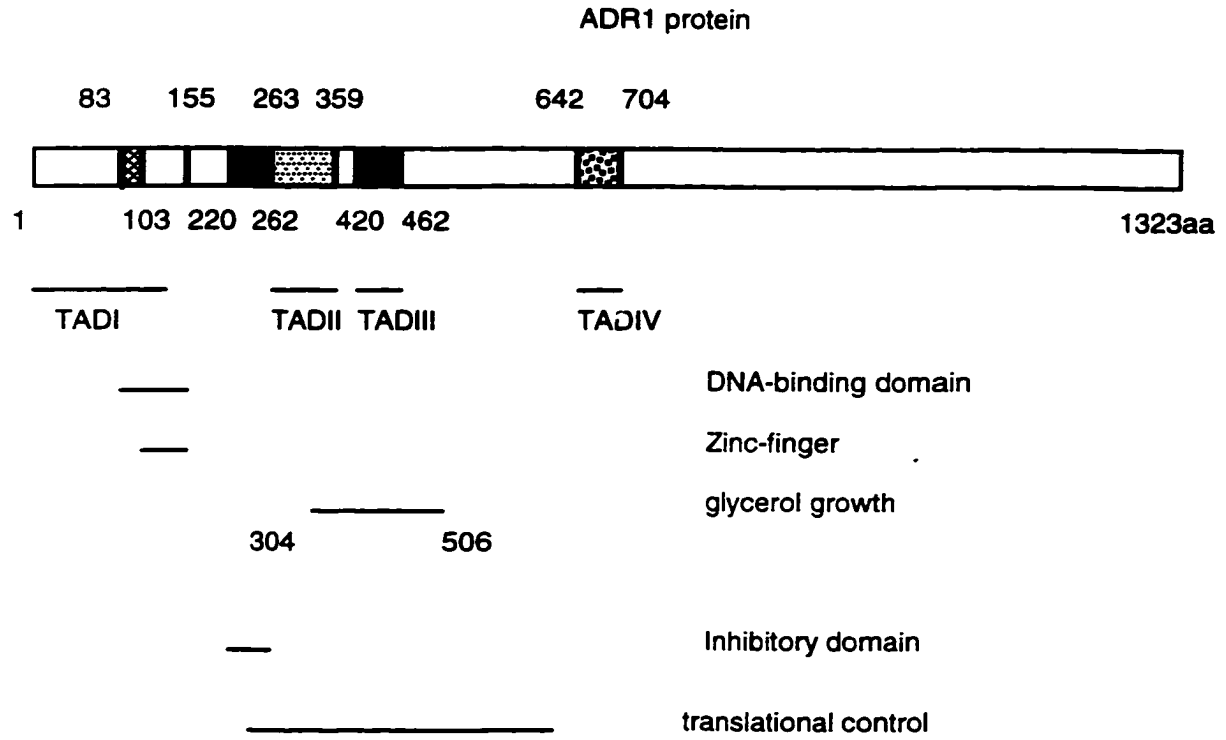

Figure 9. Functional domains of the transcriptional activator ADR1. ADR1 has a DNA-binding domain with 2 zinc-finger motifs. Four activation domains have been identified in the N-terminal region and extend from residues 148-242, 262-359, 420-462, and 642-704 respectively. A domain related to inhibiting ADHII activity is located at residues 220-262. A sequence overlapping TADII and TADIII is characterized as a domain regulating growth on glycerol. ADR1 expression is mostly regulated at the translation level and this protein level control appears to be mediated by the residues 262-642.

mini-ADR1 containing only the DNA binding domain of ADR1 and TADIII is capable of substituting for full-length ADR1 using a UAS1-containing reporter genes, deletion of this particular TAD (residues 420-462) in wild-type background causes no significant reduction in ADH2 expression (Cook et al 1994a). In addition, TADII and TADIV can also support transcription activity of ADR1 for growth on ethanol and /or glycerol. So multiple TADs of ADR1 are still required for the full expression of ADH2 and certainly many peroxisome and oleic acid metabolism genes.

ADR1 has been shown to interact with components in the TFIID and SAGA complex and at least several interactions appear physiologically relevant to ADH2 expression (Chiang et al 1996). TFIID in yeast consists of TBP and 12 TBP associated factors (TAF_{II}s) (Table 2). TBP is required for all three RNA polymerases (Cormack et al 1992, Tansey et al 1997). Ribosomal promoters typically have intrinsic curvature while TBP is responsible for conferring curvature on to RNA polymerase II promoters (Schatz et al 1997). The approximate 45°C bending of TATA-box induced by TBP binding, however, does not disrupt the Watson-Crick base-pairing or cause a conformational strain (Schatz et al 1997). A consequence of this convex structure is that the upper surface of TBP exposed and available for protein-protein interactions. The major contact of TBP with

Table 2. Yeast, drosophila and human TFIID components and yeast SAGA

SAGA TFIID yeast	TFIID Drosophila	TFIID human	features
TBP	TBP	TBP	
GCN5 TAF130/145	TAF230/250	TAF250/CCG 1	HAT kinase activity for TAF230 and TAF250
	TSM1/TAF150	TAF150	cell cycle (G2/M)
AF90 TAF90	TAF80	TAF100	cell cycle (G2/M) WD-40 repeats
TAF68 TAF68/61	TAF30α/28/22	TAF20/15	Histone H2B motifs
TAF67	TAF55	TAF55	
TAF60 TAF60	TAF60/62	TAF70/80	Histone H4 motifs
TAF47			
TAF40	TAF30β	TAF28	
TAF30/ ANC1/TFG3 /SWP29			Component of TFIIF
TAF25 TAF25/23		TAF30	
SPT3 TAF19/ FUN81		TAF18	
TAF17 TAF17/20	TAF40/42	TAF32/31	Histone H3 motifs
ADA1 ADA2 ADA3 SPT7 SPT8 SPT20			

References:

Apone et al 1996; Henry et al 1994; Moqtaderi et al 1996; Roeder et al 1996; Tansey et al 1997; Walker et al 1996; Grant et al 1998; Lee et al 1998

TATA element is a hydrophobic contact between monomer TBP and the minor groove (Horikoshi et al 1990). The major groove, however, also contributes to the full function of the promoter (Burley et al 1998). TBP binding TATA-box is essential for basal transcription (Poon et al 1991). However, TFIID rather than TBP is the functional entity in vivo. TBP or TFIID binding to the promoter is enhanced by transcriptional activators and GTFs or reduced by repressors (Li et al 1999, Kuras et al 1999).

The TAF proteins may mediate the interactions of transcription activators with the general transcriptional machinery. Several lines of evidence suggest that TAFs play a variety of roles, such as a cofactor/mediator and in cell cycle progression. TAFs also contribute to the recruitment or induction of an active RNA polymerase II through interacting with the GTFs at least at some promoters (Chen et al 1994, Verrijzer et al 1994). However, it is still under much debate and investigation whether the TAF proteins play a general role in transcription (Reese et al 1994, Walker et al 1996). TAFs function as coactivators or corepressors at many genes and associate with the core promoters (Reese et al 1994, Manley et al 1996). To date the TAFs that share histone motifs appear to play a broad role in gene transcription while other TAFs are only required for a small set of genes (Apone et al 1998,

Michel et al 1998, Natarajan et al 1998). For instance, TAF40 and TAF90 only affect a set of genes (Klebanow et al 1997). For TATA-less promoters, TBP-TATA binding is dispensible while TAFs are required for basal transcription (Martinez et al 1994). Though each of the TAF proteins do not play a general role in transcription, they are essential for cell viability (Poon et al 1995).

Two large size TAF proteins, TAF150 and TAF130, are essential for a number of cyclin genes and may play roles in promoter selection and downstream contacts (Nishikawa et al 1997). TSM-1/TAF150 is required for G2/M phase progression. In addition, the drosophila TAF150 homolog (C-terminal 489 aa) binds to a promoter element named initiator element (Inr). Inr is pyrimidine-rich (YYA+1N(T/A)YY) and is located around the transcription start site (Verrijzer et al 1994). This Inr is especially important for TATA-less promoters and recognition of Inr by dTAF150 is required for activation of at least some promoters (Hansen et al 1995).

TAF130/145 is well conserved from yeast to human and its homologs in drosophila and human have been isolated and characterized (Ruppert et al 1993). Like its homologs, TAF130 in yeast possesses HAT activity (Mizzen et al 1996). This protein has been structurally characterized and the essential region includes residues 208-303 and

367-1037 (Bai et al 1997, Figure 10). The N-terminal residues 2-115 of TAF130 mimics the hydrophobic minor groove face of a TATA element and binds TBP competing with TBP's interaction with the TATA-box (Burley et al 1998). This domain can actively dissociate TBP from TATA –box promoters in vitro and this negative effect is physiologically important (Kotani et al 1998). However, other regions of TAF130, such as the region between the conserved essential domains and the HMG (high-mobility group), are also required for TBP interaction (Kokubo et al 1994). TAF130 might also compete with activators for binding TBP (Nishikawa et al 1997). Microarray analysis reveals that inactivation of TAF130 only elevates the transcription of around 1% of total genes in yeast and this effect is independent of its cell cycle effect (Holstege et al 1998). However, this is a very conservative estimate of TAF130 effects on gene expression because these effects were only quantitated following growth on rich medium. As the only known TAF protein that contacts TBP directly, TAF130 also participates in promoter selection and its activity is core promoter-dependent (Shen et al 1997). This role does not require upstream activating sequences but requires the sequence around the TATA-box. Its role in promoter selection may involve recruiting other TAF proteins.

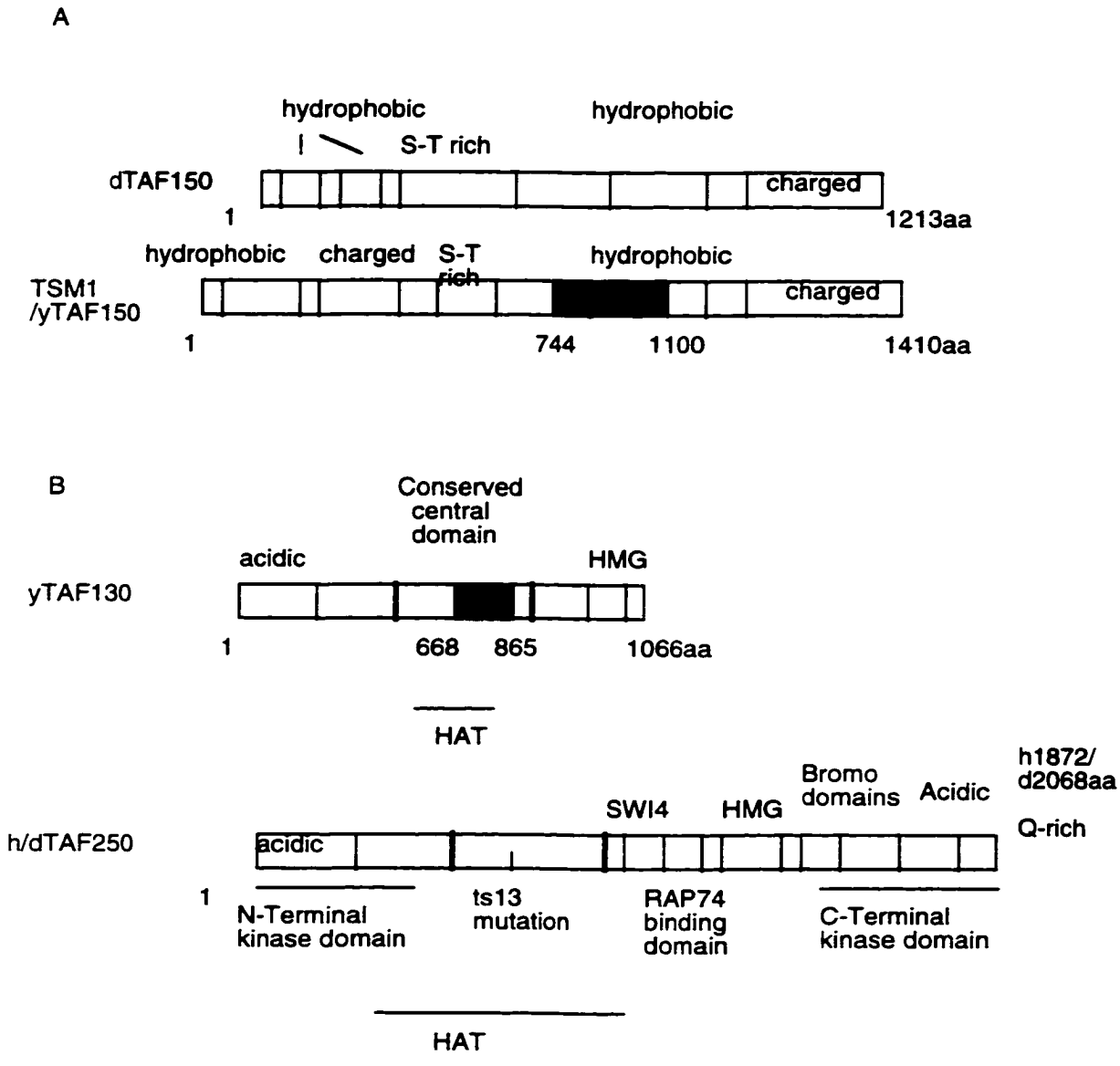

Figure 10. Comparison of yeast TAF130 and TAF150 proteins with their homologs in drosophila and human. References are Bai et al 1997, Verrijzer et al 1994 and Mizzen et al 1996. Regions interacting with TADIV of ADR1 are highlighted.

Histone motif-containing TAF proteins such as TAF60 (H4-like), TAF61/68 (H2B-like), and TAF17 (H3-like) appear to play broad roles (Michel et al 1998, Natarajan et al 1998 and Holstege et al 1998). Tight ts mutations in these TAFs confer significant reduction in total poly A+ RNA and several tested mRNAs. So these TAFs are likely to be required at most genes. The broad roles of these TAFs have been suggested to related to the fact that they are also part of SAGA complex (Apone et al 1998). But some evidence suggest that its broad role is not related to their roles in the SAGA. Disruption of SAGA caused by deletion of the SPT20 subunit does not result in a general reduction in mRNA (Michel et al 1998). It has been suggested that these histone-like TAF proteins increase TFIID affinity for the promoter through forming a histone-octamer-like structure.

TAF61 is required for SAGA integrity and the HAT activity of the whole complex. It has been shown to mediate the interactions of the transcriptional activators like GCN4 with the SAGA complex (Natarajan et al 1998). The drosophila homolog of TAF17 contacts both TFIIB and the upstream activator. TAF17 is required by a number of genes (Apone et al 1998) and DNA microarray analysis demonstrated that a TAF17

defect affected 67% of the total 5349 transcripts tested (Holstege et al 1998).

Previously, it has been demonstrated that ADR1 requires the TFIID complex for activating ADH2 gene (Komarnitsky et al 1998b). TADI and TADIV also interacted in vitro with the C-terminal region of TFIIB while TADII of ADR1 could bind ADA2 and all four TADs interacted with another SAGA component GCN5. Most importantly, it has been shown that deletion of the GCN5 or ADA2 gene affects the nucleosome positioning and the transcription activation of ADH2 upon glucose depletion (Caserta, M.. personal commun.). TFIID components also interact with TADII and TADIV and these interactions appear physiologically relevant to the ADH2 gene activation. Both TADII and TADIV interact with TAF130 and TADIV also binds several other TAF proteins (Komarnitsky et al 1998b). A mutation in TAF130 was also found to affect the transcriptional activity of LexA-ADR1-TADIV. In addition, ADR1 was shown to co-immunoprecipitate with TFIID components in crude protein extracts.

A systematic binding study was therefore conducted to determine which TAF proteins interact with TADs of ADR1. Twelve TAF proteins were expressed in vitro and examined individually for binding TADs. These

interactions were confirmed using purified TAF proteins in the binding assay. Furthermore, the regions of TAF150 and TAF130 that bound TADIV were localized. To examine the physiological relevance of these interactions, effects on ADH2 expression of inactivation or mutation of these TAFs or upon TFIID disruption were determined using Northern analysis and S1 nuclease assay. Inactivation of different TAF proteins exhibited distinct effects on ADH2 transcription, from no effect to a significant effect. Different sets of TAF proteins may be required at different promoters and at one promoter, such as ADH2, they may act synergistically by sequential or conditional interactions with the transcriptional activators.

Material and Methods

Yeast Strains And Growth Media

Yeast strains are listed in Table 3. BYΔ2 strains were grown on YP rich medium supplemented with 2% ethanol/2%glycerol. All other TAF mutation strains were first grown on YP medium with 4% glucose to OD_{600} 0.5 and then shifted to 2% ethanol/2% glycerol. Cells were washed once and after washing no glucose was detectable using a glucose strip

Table 3. ADR1-related yeast strains

Strains	Genotype
BYΔ2/WT-TBP	MATa ura3-52 ade2-101 trp1-Δ1 lys2-801 GALΔleu2 ΔSPT15 YCP22-TRP1-TBP
BYΔ2/K151L,K156Y	same as above except YCP22-K151L K156Y
A893	MATa ura3 ade1 his4 leu2 tsm1(ts)
YSW87	ura3 taf145::LEU2 pTAF130/145:HIS3
YSW90	isogenic to YSW87 except ptaf130/145-ts1
YSW93	isogenic to YSW87 except ptaf130/145-ts2
TAF60	MATa ura3-52 TRP1 leu2 LEU2::taf60 pRS313-HIS3-TAF60
TAF60-12	isogenic to TAF60 except pRS313-HIS3-TAF60-12
TAF60-19	isogenic to TAF60 except pRS313-HIS3-TAF60-19
TAF61-Δ1	MATa adh1 ura3-52 trp1-Δ1 leu2::pET5b LEU2::taf61 pRS313-HIS3-TAF61
TAF61-12-Δ1	isogenic to TAF61-Δ1 except pRS313-HIS3-taf61-12
TAF61-23-Δ1	isogenic to TAF61-Δ1 except pRS313-HIS3-taf61-23
TAF61-108-Δ1	isogenic to TAF61-Δ1 except pRS313-HIS3-taf61-108
TAF61-226-Δ1	isogenic to TAF61-Δ1 except pRS313-HIS3-taf61-226

test. These strains were then grown in YP medium containing 2% ethanol/2% glycerol for another 1.5 to 2 hrs at 30°C and then moved to 37°C for another 1.5 to 2 hrs.

DNA Constructions

TAF130/145-668-865 was constructed by Yeuh-Chin Chang (University of New Hampshire). pSPTAF130 was cut with EcoRI and HindIII and the small fragment was ligated to the pET23d T7 vector digested with the same enzymes. The construct was checked by digestion with the two enzymes, and in an in vitro expression system only one protein band running around 20 KD was observed. Two T7 vectors were constructed for expressing the N- and C- terminal of TAF40 proteins in vitro. pET-15b-TAF40 was cut with NheI and the larger fragment was self-ligated to form the TAF-40-N (1-466 nucleotides) vector, expressing the N-terminal 1-155 aa of TAF40. To construct TAD40-C, pET-15b-TAF40 was cut with NheI and BamHI and the small segment was ligated to pET-28a-c(+) plasmid digested with the same two enzymes. TAD-40-C without the T7 tag contains nucleotide 466 to 1024 or 466 to 1041 and expresses amino acids 156-340 or 156-346. Based on the restriction maps of pET-28a-c(+) and TAF40 gene, if the BamHI cut at 1041 nucleotide, the C-terminal 6His tag is not in frame and if the enzyme cut

at 1024 nucleotide, the C-terminal 6His will be expressed. Both the N-terminal and C-terminal constructs were expressed in vitro using a wheat germ extract and a single protein band was observed for each when translated in vitro.

ADHII Enzyme Assay

Native gels were run as described previously (Williamson et al 1981). Protein levels were measured using Bradford assay and equal amounts of protein (25 ug or 50 ug) were loaded into each lane. PAGE gels were run overnight at 4°C.

In Vitro Binding Assay

The procedure for binding ^{35}S-Met labeled proteins was conducted as described in detail (Chiang et al 1996). T7 and SP6 Promega wheat germ kits for in vitro expression were adopted for different TAF proteins. GST fusion proteins to ADR1 TADs I to IV and TADIV deletion constructs were overexpressed and purifed from E. coli cells (Chiang et al 1996). Purifed TAF150, TAF130, TAF90, TAF60, and TAF61 proteins were a gift from Dr. P. Anthony Weil. The ECL-based detection system was used for Western analysis of TAF proteins and all antibodies were also gifts from Dr. P. Anthony Weil and diluted as instructed.

S1 Nuclease Analysis

S1 nuclease analysis was conducted as described (Jackson-Fisher et al 1999). The exception is that 10 nucleotides were added to the 3' end of the ADH2 probe. The sequence is 5' GGAGCA CTTTGC ACCGGC TGGCAA ACCAAC CAAGAC AACAGT ACCGTT CGCCCT ACAG 3'. The controls were two tRNA probes (isoleucine and tryptophan) synthesized as described (Cormack et al 1992). [γ-^{32}P]ATP was from ICN and the specific activity was 2000 uci/ 12 ul.

Results

Transcriptional Activation Domains of ADR1 Bind to only Certain TAF Proteins.

Previous studies have shown that TAD domains of ADR1 can retain TAF components of TFIID from the yeast extract. Therefore, a systematic survey was conducted to investigate which individual TAFs of the total of 12 present in TFIID were capable of binding TADIV of ADR1. We expressed TADIV as a GST-fusion protein and used it to individually bind in vitro expressed TAF proteins. A total of five TAF proteins, TAF150, TAF130, TAF61, TAF60 and TAF40, were found to bind TADIV and the binding results of TAF150, TAF130 and TAF60 which I studied are shown in Figure 11. Approximately 2 to 5% of the total input protein for each of these TAFs was found to bind TADIV. In addition to using GST as a

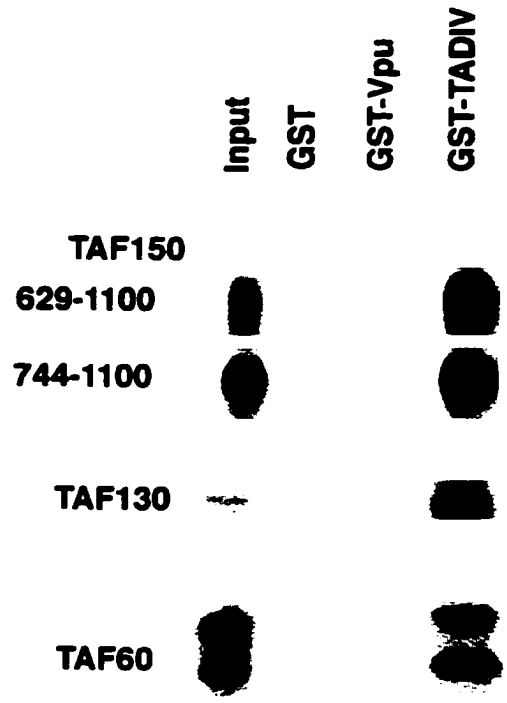

Figure 11. In vitro translated TAF proteins bind TADIV. All TAFs were expressed in vitro using a Promega wheat germ expression kit. all TAFs except for TAF150 were full length in size. GST, GST-VpU and GST-TADIV were expressed in E. coli. and purified using glutathione beads obtained from Sigma. Equal amounts of GST fusion proteins were used to bind the TAF proteins individually. Inputs were all 5% of the amount used for the binding except for TAF130 that was 2% of the total proteins.

negative control, GST-Vpu, an HIV AIDS viral protein was also used as a negative control in which case little or no binding was observed to the in vitro translated TAF proteins. All TAF proteins were full-length except in the case of TAF150. For TAF150, two truncated overlapping fragments were analyzed and both were capable of binding TADIV. However, this does not exclude the possibility that other regions of the large TAF150 protein also interacted with TADIV. The residues 744-1100 of TAF150 that bind TADIV is located in a non-conserved region of the protein (Figure 10). Therefore, a total of five TAF proteins interacted with TADIV of ADR1.

We also analyzed the ability of other ADR1 TADs to bind to twelve TAFs. TADI and TADII were found to bind TAF130, TAF60 and TAF61 (Figure 12, data not shown for TAF61 whose binding assay was done by Karen Reed, who was a postdoctoral fellow in our lab). In vitro expressed TAF130 displays multiple bands below the full-length sequence of 130 kD, indicating that there exists either degradation products or mistranslation peptides from internal methionines. These truncated products were capable of binding much better to TADII or TADI when compared with full length TAF130 (data not shown). TAF60 and TAF40 also specifically bound TADII (Figure 12 and Figure 13). Considering

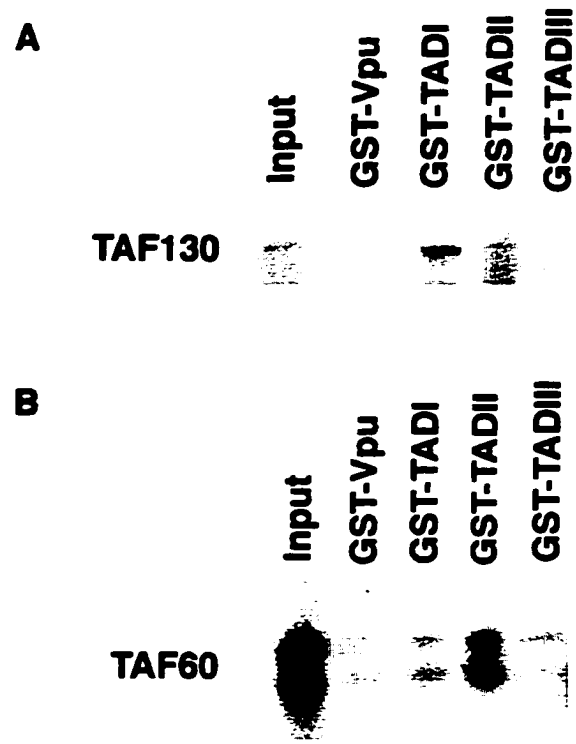

Figure 12. Binding of TADI, TADII, TADIII to TAF130 and TAF60. The same binding procedure as described in Figure 11 legend was used for the binding. Purified GST-VpU, GST-TADI, GST-TADII, and GST-TADIII proteins were examined with Coomassie blue stained SDS-PAGE gel and equal amounts were used for the in vitro binding assays.

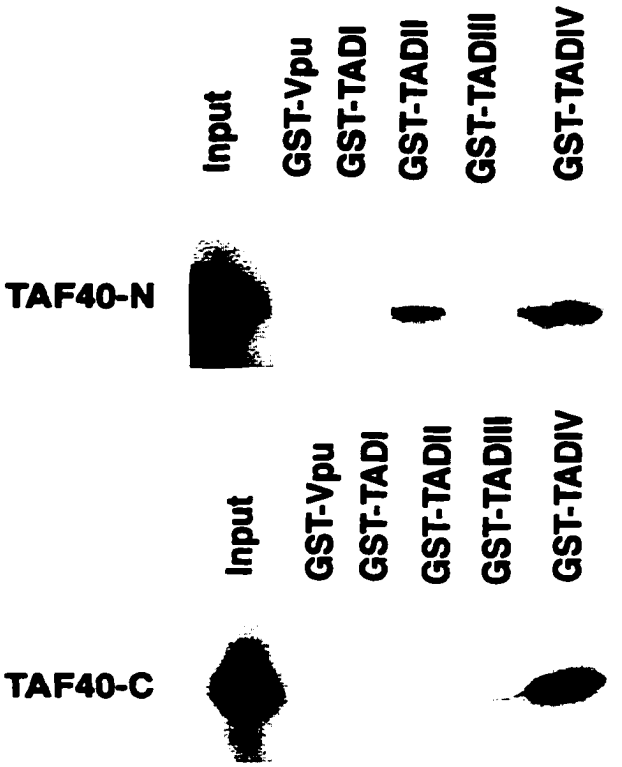

Figure 13. Binding of TAF40 N-terminal and C-terminal domains to TADI to IV. DNA constructions of TAF40 were described in the Material and Methods. There are 3 input lanes for the TAF40 N-terminal binding. Each input lane was 5% of the total amount of proteins used for binding.

TAF150 is huge protein, it is possible that other regions of TAF150 may interact with TADI and/or TADII, too. In contrast to the multiple interactions of TADII with TAF proteins, TADIII bound none of the 12 TAF proteins.

To locate the region of TAF40 that bound TADIV, two DNA constructs were made to express the TAF40 N-terminal (residues 1-155) and C-terminal (residues 155-341 or 346), respectively. Both of these TAF40 proteins were found to physically interact with TADIV whereas only the N-terminal TAF40 protein interacted with TADII (Figure 13). Combined with the above data, it is apparent that multiple TADs in the transcriptional activator ADR1 interact with multiple TAF proteins, indicating that there is a redundancy and/or coordination in the interaction of ADR1 with TFIID components.

Purified TAF Proteins Bind TADIV

Because TADIV had been more well defined than the TADI or TADII, the subsequent analysis using purified TAF proteins was focused on TADIV interactions. Purifed TAF proteins were used to bind TADIV in order to confirm the in vitro TAF binding results described above. Purified full length TAF150, TAF130, TAF90, TAF60 and TAF61 all were found to bind TADIV (Figure 14, Bai et al in prep). Purified TAF40, however, did

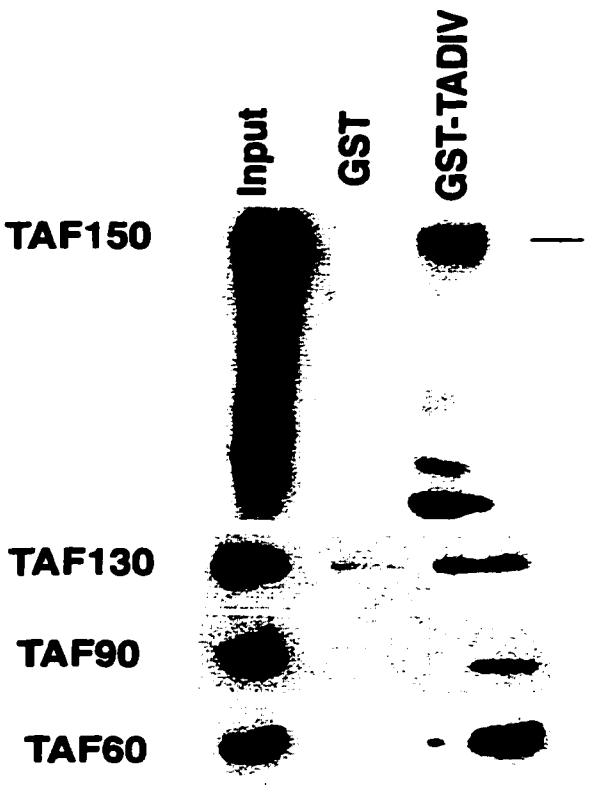

Figure 14. Binding of purified TAF proteins to TADIV. The TAF proteins that have been shown to bind TADIV as described in Figure 11 were examined in their purified form for their ability to bind TADIV. Instead of using truncated versions of TAF150, full length TAF150 was used. Inputs were 5% for each protein and ECL was used to detect the protein following incubation with the proper antibody.

not bind TADIV (K. Reed, pers. comm.). Therefore, a total of four TAF proteins, TAF150, TAF130, TAF60, and TAF61, bind to TADIV in either in vitro translated form using wheat germ extract or in purified form.

Residues 668-865 of TAF130 Alone Bound TADIV.

Since TAF130 is the most extensively studied TAF protein, our subsequent studies focused on its binding to TADIV. A series of TAF130 deletion constructs that covered the whole sequence of TAF130 was used to identify the region of TAF130 protein that was responsible for binding TADIV (Figure 15A). Three TAF130 deletion constructs (Δ12, Δ13, Δ14) exhibited significant reduction in binding TADIV among the 17 deletion constructs analyzed (Figure 15A, lane 3). In constrast, TADII bound these three TAF130 deletion proteins to the same extent as other TAF130 deletion constructs (lane 4). These results suggest that residues 692-860 represent the region that ADR1 TADIV interacts with TAF130. To confirm this hypothesis, residues 668-865 of TAF130 were placed under T7 promoter control in order to express TAF130-668-865 in vitro. Binding studies with this expressed protein showed that by itself it was capable of binding TADIV (Figure 15B). Therefore, the residues 668-865 are responsible for the interaction of TADIV with TAF130 and this region is located in a well conserved region in the TAF130 protein (Figure 10).

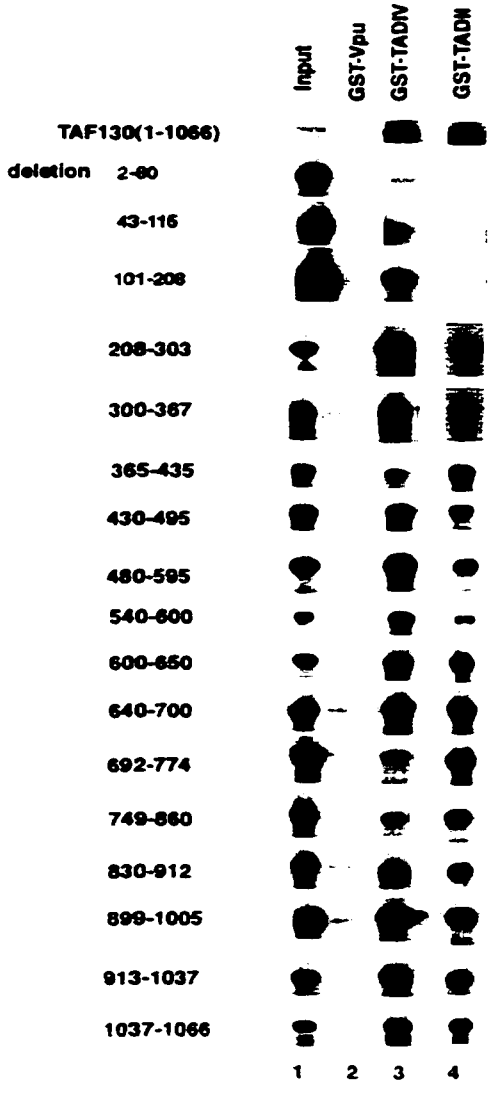

Figure 15A. Binding of TAF130 deletion proteins to TADIV. All TAF130 deletion constructs were expressed in vitro under an SP6 promoter. Each of these TAF130 deletion proteins was used for binding GST-VpU (lane 2), GST-TADII (lane 4) and GST-TADIV (lane 3). The input was 5% of the total protein used for binding.

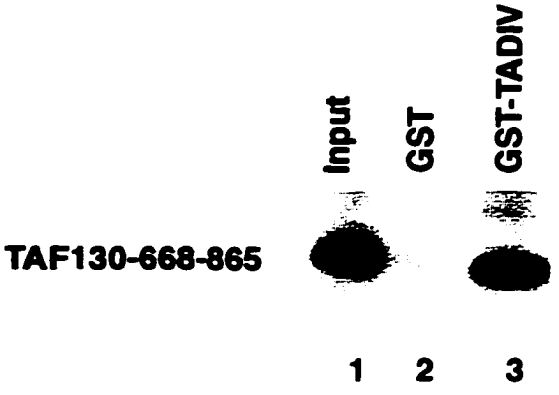

Figure 15B. Binding of residues 668-865 of TAF130 with GST-TADIV. The region of TAF130 corresponding to amino acids 668 to 685 was put under control of a T7 promoter and expressed using an in vitro expression system as described in Figure 15B. Five percent of total protein used for binding was loaded into the input lane.

Residues 698-704 Of TADIV Are Essential For TAF Protein Interaction As Well As For Transactivation.

It has been shown that deletion of residues 698-704 dramatically affect the transactivation ability of LexA-TADIV (Kormanitsky et al 1998b). To determine how deletions within TADIV protein affects the ability to bind individual TAFs, all four relevant TAF proteins that were capable of binding TADIV were used for binding a series of TADIV deletion proteins as described (Komarnitsky et al 1998b). Biochemical data as shown in Figure 16A demonstrated that residues 698-704 were essential for TADIV binding to TAF150, TAF130 and TAF60. Previously, Karen Reed, a postdoctoral fellow in our lab, also showed that TAF61 exhibited a similar binding pattern with the TADIV deletion constructs (Bai et al in prep). Therefore, all four TAF proteins that bind to TADIV require the 698-704 residues in the TADIV.

Mutation analysis of TADIV was conducted to identify residues that may be essential for binding each TAF protein and for transactivation of a reporter gene. Mutation analysis in the region of residues 675 to 704 revealed that certain residues were essential for the transactivation of the reporter gene ß-galactosidase. As shown in Table 4, all of the mutations affected the transactivation by two fold or less except F685A

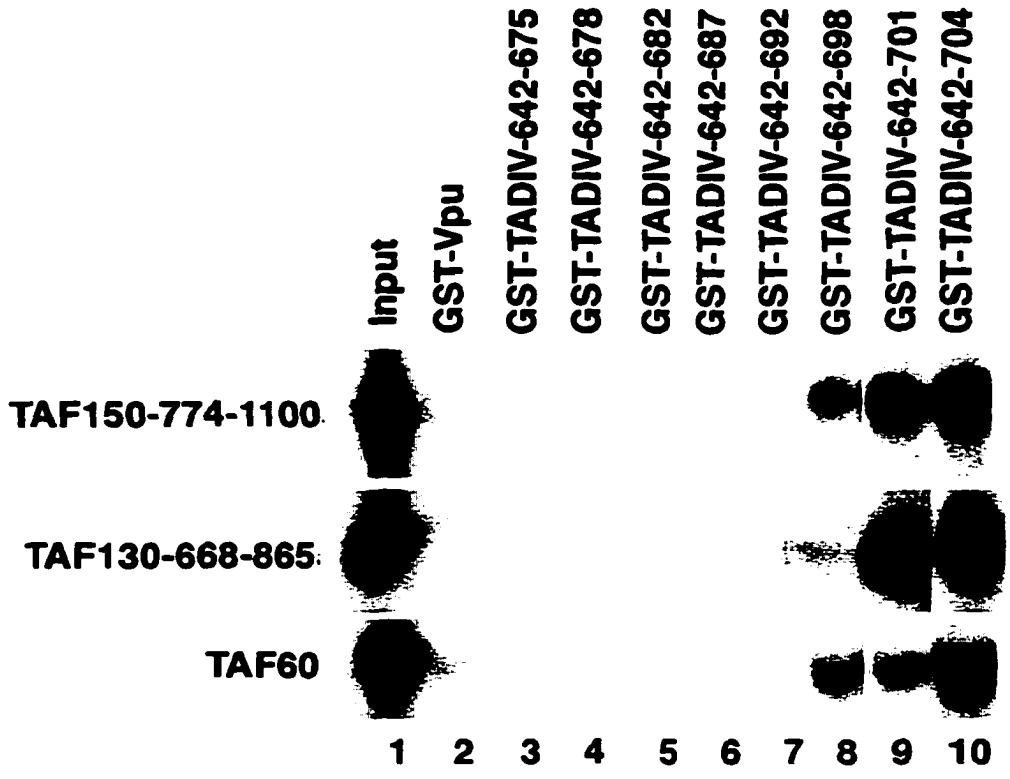

Figure 16A. Binding of TAF150, TAF130 and TAF60 to TADIV deletion proteins.

Table 4. Effects of mutations on LexA-TADIV's trans-activity

mutations	TADIV WT	L684A	F685A	L688A	E695A	Y696A	D697A	I703A	L704A
β-galactosidase activity (U/mg)	366	224	84	232	180	186	150	191	118

188/34 strains carrying the LexA-TADIV or LexA-TADIV mutations were grown on 2% galactose/ 2% raffinose and the β-galactosidase activity was assayed as described (Kormanitsky et al 1998). The values represent the average of more than 7 times assays.

which reduced the β-galactosidase activity much more significantly. Two of these mutated TADIV constructs were analyzed to correlate their trans-activity with their capability of binding to the TAF proteins. As shown in Figure 16B, while the mutation I703A did not alter TADIV binding to any of the TAF proteins, the mutation F685A caused severe reduction in binding all four TAF proteins. These results suggest that the hydrophobic residue at 685 may be important for TADIV function because it affected binding to all four TAFs. Alternatively, it could also be important for maintaining the three-dimensional structure of this region.

Inactivation Of TAF130 Did Not Affect ADH2 Transcription and TAF150 Might Have An Effect On ADH2 Transcription When the Diauxic Shift Occurs.

Because most of the above experiments solely represent biochemical interactions, it was also important to explore the physiological role of these TAF proteins in ADH2 expression. Wild-type and TAF mutation strains were analyzed for their effects on ADH2 mRNA levels and ADHII enzyme levels. For TAF130 two temperature sensitive (ts) strains carrying separate TAF130 ts mutations were used for the study. All strains were pregrown in 4% glucose and then shifted to ethanol (diauxic shift). There was no significant alteration in the ADHII enzyme activity for

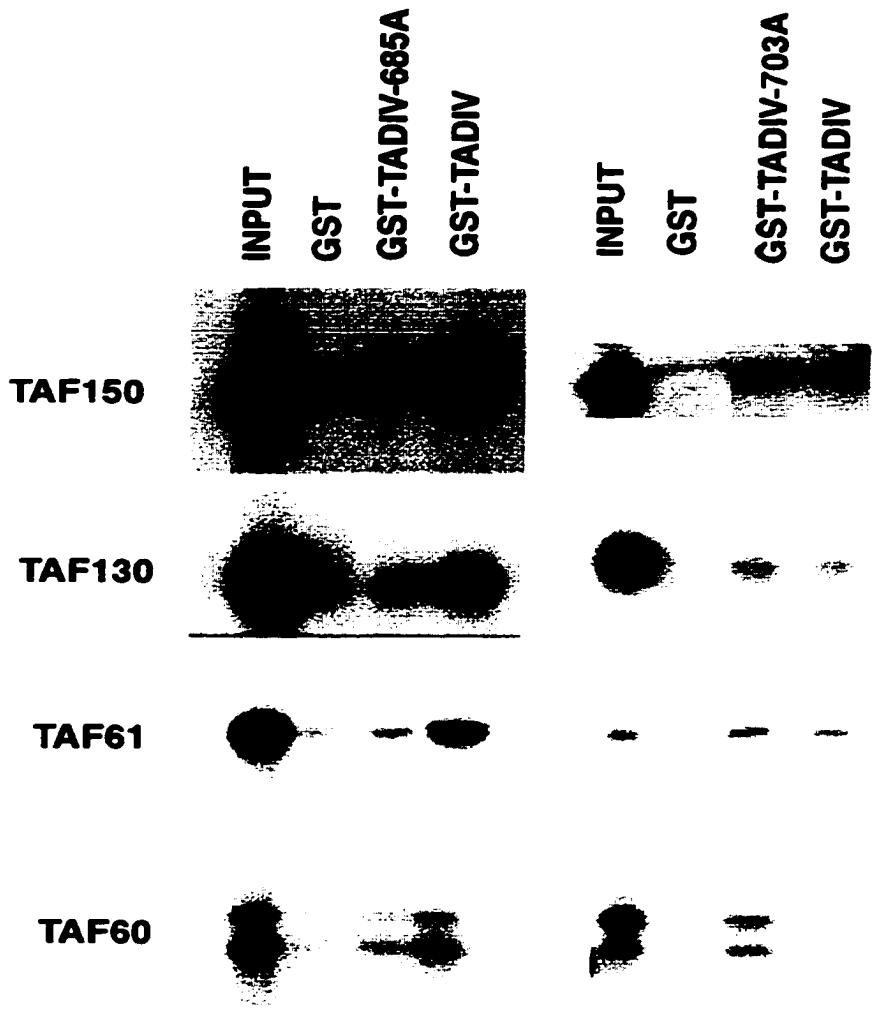

Figure 16B. Binding of TAF150, TAF130, TAF61, and TAF60 to TADIV-F685A and Y703A and to wild-type TADIV. Deletion or mutation constructs extending from residues 675 to 704 were expressed as GST fusion proteins. Equal amount of these proteins were used to pull down the in vitro radioactively-labeled TAF150, TAF130, TAF61 and TAF60 proteins, respectively.

all strains and conditions tested (Figure 17A) . Also, when the total RNA was subsequently isolated from each strain and analysed by Northern analysis, there was no dramatic change of ADH2 transcripts in the wt as compared to the TAF130 ts strains (data not shown). It is necessary to point out that all TAF130 strains are abnormal when compared with other stains we work with. Unlike other wild-type strains that grow very well on 2% ethanol /2% glycerol at 30°C, we observed that they grow very poorly possibly due to lack of ADH2 expression. Therefore, TAF130 might not have dramatic effect on ADH2 expression.

TAF150 strains were also grown on 2% ethanol or by depleting glucose and ADHII enzyme assays were conducted on TAF150 protein extract. There was no significant alteration of the ADHII enzyme activity when tsm-1/ taf150 strains was shifted to restrictive temperature (Figure 17B). When the total RNA extract were subjected to Northern and S1 nuclease assay, there was no effects observed when the ts strains were shifted to restrictive temperature on 4% glucose or 2% ethanol (data not shown). But when the cells were subject to diauxic shift, there were dramatic reduction of ADH2 transcripts for taf150-ts strains grown at 37° compared with the strain grown at 30° (Figure 17C). Because no wild-type strains with the same genotype as the TAF150-ts strain were

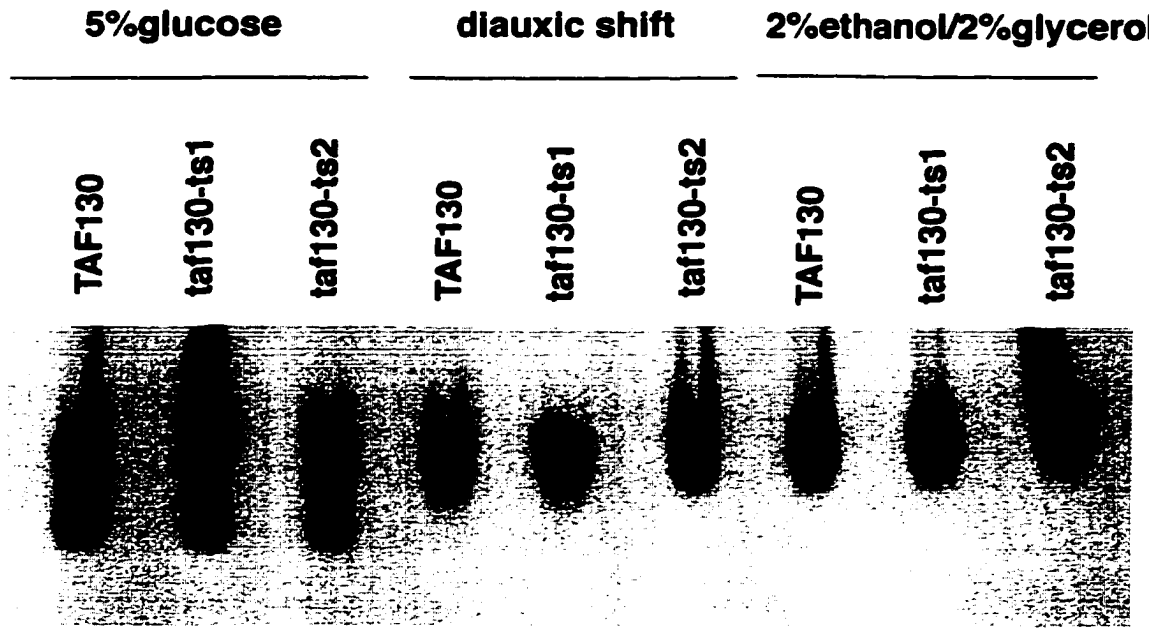

Figure 17A. ADHII enzyme assay of TAF130 wild-type and ts proteins. Total proteins were extracted from freshly collected cells and quantified by the Bradford assay. Fifty ug was loaded in each lane. YSW87 (wt), YSW90 (taf130-ts1), and YSW93 (taf130-ts2) were grown on 4% glucose, 2% ethanol/glycerol or subject to a diauxic shift.

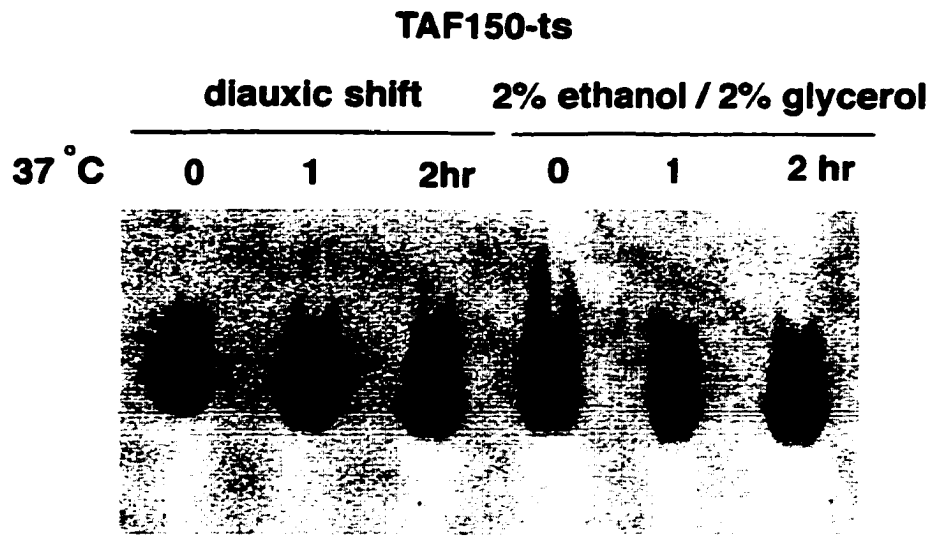

Figure 17B. ADHII enzyme assays of proteins from TAF150 and taf150-ts strains. Strain A893 (taf150-ts) were either grown on 4% glucose and shifted to 2% ethanol or on 2% ethanol continuously. Zero, one, and two hours represent the times cells were shifted to 37°C.

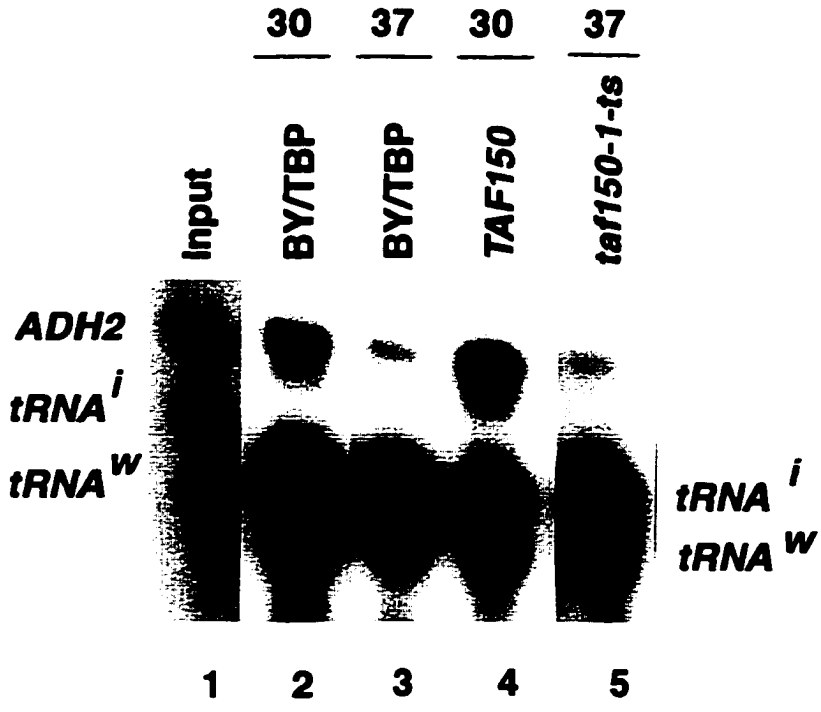

Figure 17C. S1 nuclease assay of RNA from TAF150 and taf150/tsm-1 ts strains. The strain A893 upon shifting to restrictive temperature was grown on 4% glucose to an OD_{600} 0.5 and then shifted to 2% ethanol/2% glycerol for another 2 hrs. Half of the culture was subsequently shifted to the restrictive temperature for another 2 hrs growth. The culture that had been growing at 30°C was used as the control and designated as wild-type. In addition, another wt strain (BYΔ2) was used as a control and subject to the same growth conditions as strain A893.

available, two other wild-type strains, EGY188 and BYΔ2, were used for S1 nuclease protection analysis (data not shown for 188, Figure 17C, lane 1 and 2). However, this TAF150 ts strain may not contain a mutation that is expressed as a null protein. Therefore, the inactivation of TAF150 may not necessarily inactivate the protein completely and its effect on ADH2 transcription needs further investigation.

TAF61 and TAF60 Affect ADH2 Transcription.

All TAF61 and TAF60 wild-type and ts mutation strains were examined similarly as for the TAF130 and TAF150 strains to determine whether they affected the ADH2 gene in vivo. They were first grown on 4% glucose and the cells were collected and resuspended in fresh medium with 2% ethanol/2% glycerol and allowed to grow at 30° for 2 hrs. All cultures were shifted to 37° for another 2 hrs. There was no significant changes in ADHII enzyme activity in these taf61 and taf60 backgrounds (Figure 18A). However, primary Northern analysis of the total RNA from each strain indicated that there were several-fold reduction of ADH2 transcripts in a number of these taf61 mutation background and dramatic decrease in the two taf60 ts strains (data not shown). To confirm these data, S1 nuclease protection analysis was used to quantify the ADH2

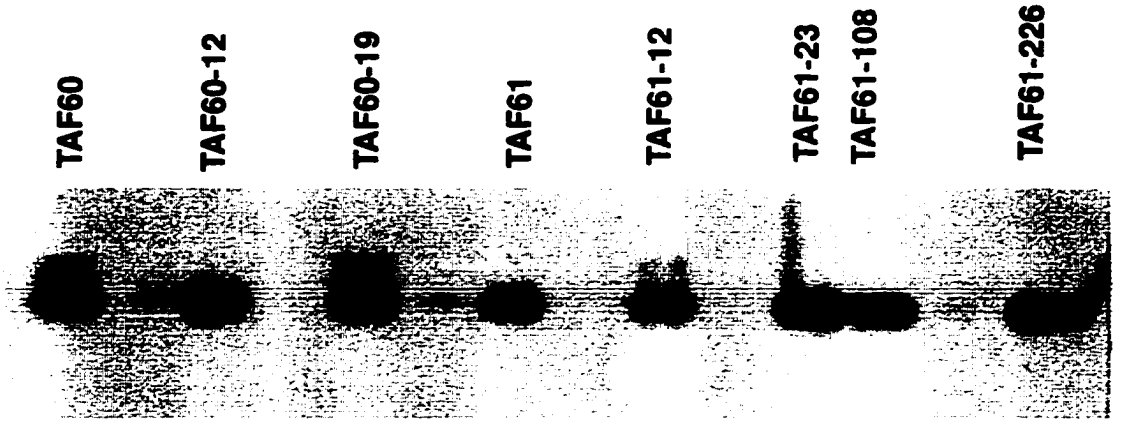

Figure 18A. ADHII enzyme assays in TAF60 and TAF61 strain and their corresponding ts strains. All strains were grown on 4% glucose to reach mid-log growth and then shifted to medium supplemented with 2% ethanol/2% glycerol for another 2 hrs. Cells were broken in native extraction buffer (Draper et al 1994) and 25 ug fresh extract was loaded for each lane.

mRNA level. As shown in Figure 18B, a several-fold reduction was observed in all taf61 ts strains except taf61-226, while dramatic decreases in ADH2 transcripts occurred in both taf60 ts strains. These data suggest that the effects at the transcriptional level do not necessarily lead to changes at the protein level due to the multilevel regulation of gene expression. Alternatively the standards used for these analyses were not representative and therefore misrepresented the true level of ADHII enzyme or RNA levels.

TFIID Disruption Affects ADH2 Transcription And Expression.

If individual TAFs are important for ADH2 transcription, the assembly of the whole TFIID should also be essential for ADH2 gene expression. To determine the effect of the TFIID structure disruption on ADH2 expression, a strain carrying a TBP mutation was used. The mutations K151L K156Y in TBP caused the disruption of the TFIID complex at the restrictive temperature of 38°C. To examine the effect of TFIID disruption on ADH2 expression, the total RNA and proteins from these strains grown on 2% ethanol/2% glycerol were analyzed. Both Northern and S1 nuclease assay data exhibited a significant reduction of ADH2 transcripts (data not shown, Figure 19A), respectively. $tRNA^{i}$ and $tRNA^{w}$ were were used as the internal controls for comparison.

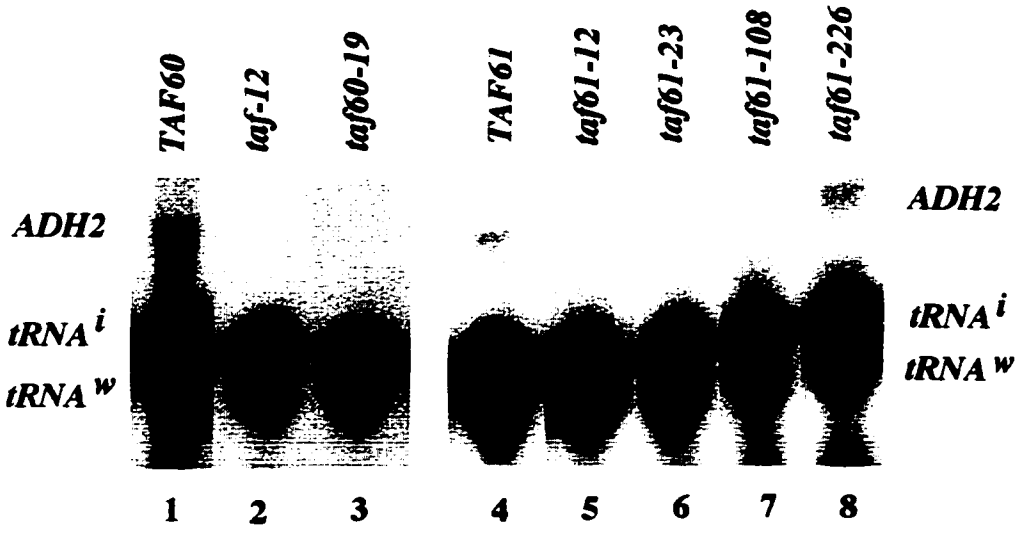

Figure 18B. S1 nuclease assay of ADH2 transcripts from TAF60 and TAF61 wt and ts strains. Yeast strains were grown as described in Figure 18A legend and were subsequently transferred to the restrictive temperature of 37°C to inactivate the relevant TAF protein. $tRNA^w$ (tryptophan) and $tRNA^i$ (isoleucine) were used as internal controls.

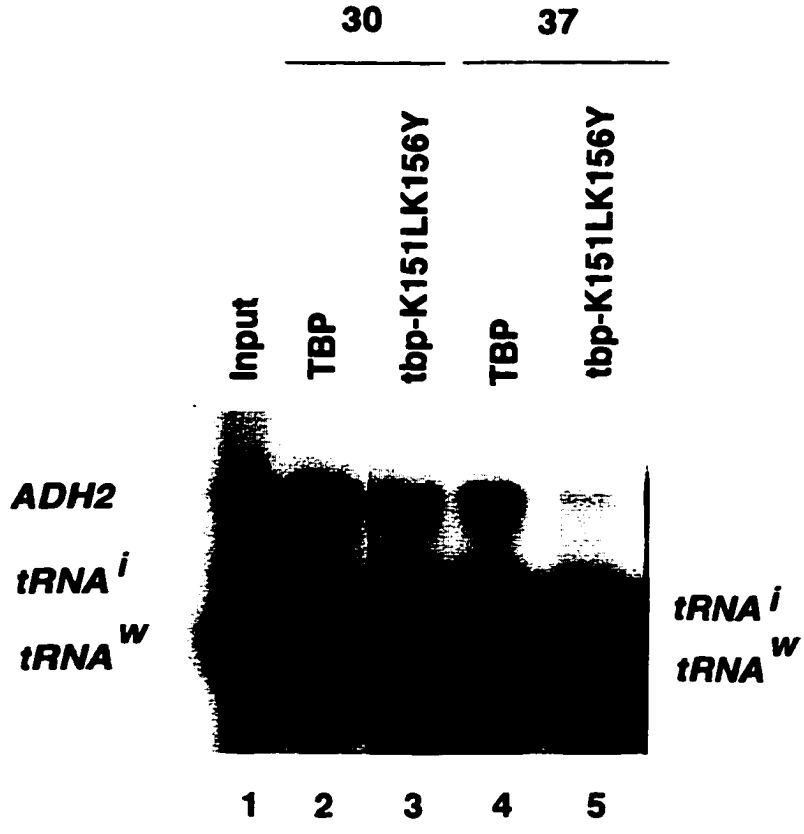

Figure 19A. S1 nuclease assay of RNA isolated from WT and TFIID disruption strains. The BYΔ2 and the strain carrying tbp K151L K156Y were grown on 2% ethanol/2% glycerol and then both were shifted to the restrictive temperature. The amount of RNA was adjusted to show equal tRNA transcripts.

Therefore, the effect shown in Figure 19A represents a minimal effect on ADH2 expression. It is necessary to point out that rRNA transcription was also severely reduced in this TFIID disruption strain and to a lesser extent, $tRNA^{i}$ and $tRNA^{w}$, when compared with the strains, the strain containing the TFIID disruption also conferred a reduction in ADH2 enzyme activity (Figure 19B). Therefore, the disruption of TFIID structure not only reduced the ADH2 transcription but also its protein expression. Considering TAF61 and TAF60 are components for both the SAGA and the TFIID complexes, this set of data also suggest that TFIID is specifically required at the ADH2 gene.

Discussion

In this work we investigated the biochemical and physiological interactions of ADR1 with TFIID. Three TADs of ADR1 were shown to interact with TAF150, TAF130, TAF60, and TAF61. Though these TAF proteins bound the same region in TADIV, their roles in vivo were distinct. When TAF130, the only TAF that displays HAT activity that is related to chromatin remodeling, was inactivated, there was no effect on ADH2 transcription. The ts mutant of TAF150 did not affect ADH2 expression following growth on ethanol, but it could have had an effect when glucose is depleted. Inactivation of TAF61 or TAF60 caused severe reduction in

Figure 19B. ADH2 enzyme assay of extract prepared from wild-type (BYΔ2) and TFIID disruption strain described in A legend. Proteins were extracted from freshly collected cells, measured with Bradford and 50 ug was subjected to native PAGE as described above.

ADH2 transcripts. TFIID disruption also conferred significant decrease in ADH2 transcription and reduction of ADHII activity at the protein level.

Multiple TAF Proteins Physically Interact With Transcriptional Activator ADR1 But Do Not Necessarily Affect The Transcription Of ADH2.

Residues 668-865 of TAF130 that itself is capable of binding TADIV is located in an essential region (residues 367-1037) of TAF130. This conserved region is required for supporting cell viability (Bai et al 1997). TAF130 also displays HAT activity that may be related to the acetylation of nucleosomes (Mizzen et al. 1996). Acetylation is thought to confer remodeling of the nuclesomes and the resultant disassembled template is simutaneouly or subsequently used for transcription. Chromatin remodeling is a requirement for transcription activation but it alone can not lead to transcription activation. For instance, spt6 mutants affect the chromatin structure of SUC2 gene but do not affect its transcription (Bortvin et al 1996). Similarly, previous studies have shown that nuclesome remodeling is required but not sufficient for ADH2 gene expression and that the transcriptional activator ADR1 is required for the chromatin remodeling at the ADH2 (Verdone et al 1996). Therefore, if TAF130 is required for the chromatin remodeling at ADH2 gene upon ADR1 binding to the UAS1, this effect may not be detected by assays

designed for detecting transcriptional changes. The observation that the taf130 mutations did not affect ADH2 expression can, therefore, be explained in four ways. Either these mutations were not specific or severe enough to affect ADR1 and ADH2 expression or that taf130 inactivation may affect only the chromatin remodeling function and therefore have no observable effect on ADH2 expression. Alternatively, taf130 inactivation has no effect on ADH2 chromatin or activation and the observed binding is artifactual. Finally, it is possible that the TAF130-TADIV interaction is important but other redundant TAF-ADR1 interactions can compensate for defective TAF130-ADR1 interactions.

The region of TAF150 that ADR1 TADIV was shown to bind to, amino acids 774-1100, is located in a nonessential and non-conserved region of TAF150. However, other regions of this huge protein may interact with TADIV and/or other TADs of ADR1. Based on Northern and S1 nuclease protection data TAF150 might be specifically required by the cells to express ADH2 when glucose is depleted. TAF150 is known to affect the cell cycle. Upon glucose depletion, cells utilize the previously excreted ethanol as an energy and carbon source via oxidative respiration and gluconeogenesis. This diauxic shift also causes the cell to arrest in the G1 phase of the cell cycle. Cross-talk between the cell cycle pathway and

adaptation to metabolic changes may be occuring through TAF150 interactions with specific proteins like ADR1 being important for this adaptation to glucose depletion.

TAF60 and TAF61 have been shown to participate in the transcriptional activation of many genes (Michel et al 1998, Natarajan et al 1998). It is not surprising that they are also required for the ADH2 gene.

Based on the physical interaction and S1 nuclease assay data, TADIV interactions with multiple TAF proteins can be explained in at least three ways. First, each individual TAF interacts with TADIV in response to the different upstream transduction signals and only one or two TAF proteins interact with TADIV at one time. Second, sequential interactions of individual TAF with TADIV could lead to coordinate transcriptional activation, starting with nucleosome remodeling and followed by promoter binding and mediation of activation. Third, different residues in TADIV might be required for the contact with each individual TAF protein. To investigate these possibilities, in vitro transcription assay and mutagenesis of the TADIV region will help to exam these hypotheses.

Multiple Activation Domains Of ADR1 Participate In The Transcription Activation Of ADH2 Gene.

Though our study is mainly focused on TADIV interactions with TFIID

components, TADII also interacts with TAF60, TAF61, TAF130, and possibly, TAF150. Some of these interactions may be redundant but these multiple interactions probably act synergistically. Redundant activation domains are beneficial for cell survival. Mutations or inactivation of one activation domain will not significantly affect the transcription activity. ADR1 is presumed to interact with other GTFs as shown in the model (described in Figure 20). It has also been shown that TADI, II, IV interact with TFIIB and with SAGA components ADA2 and GCN5. Interactions of ADR1 with TFIID components might also involve TADI and other regions of ADR1 not yet characterized such as the inhibitory domain extending from residues 227 to 239. A number of mutations in this domain (ADR1^{C}) cause constitutive ADH2 expression on glucose without affecting ADR1 expression (Denis et al 1986).

Multiple Contacts Between The Transcription Activator And TFIID Is Typical.

Numerous previous studies have shown that one transcriptional activator can interact with multiple mediators or coregulators. For instance, VP16 makes specific protein-protein contacts with TFIIB,TFIIH, TAF40, TAF32, TBP and the RNA polymerase holoenzyme (Nishikawa et al 1997). GAL4 protein interacts with TBP, TFIIB, ADA2, GAL11, SRB2,

SRB10 and SRB11, and other mediator components (Melcher et al 1995, Chang et al 1997, Hirst et al 1999). Mutation in one of the non-essential mediator genes only partially compromises the activation of GAL4. Similarly, yeast GCN4 activator has two redundant activation domains. A single activation domain may act in several steps in the overall process of activation. GCN4 protein binds TAF19, TAF60, TAF90 and ADA2 and ADA3. In addition, it also interacts with SRB mediator components, SRB2, SRB4 and SRB7, independently of the TFIID and SAGA components (Drysdale et al 1998). All these activators have multiple activation domains and their redundancy may have an evolutionary benefit. In the case of TFIID contacts, multiple interactions between activator domains and TAFs can strongly increase the binding of TFIID to the promoter and coordinately activate transcription (Kuras et al 1998). Multiple contacts also contribute partially to promoter selectivity as well as the specificity of the DNA-binding domain of the activator. In addition, one activator may be required at a variety of genes so some of these interactions with a particular GTF may be required for some promoters while the other interactions at other promoters. Therefore different activators may require distinct sets of coactivators (Chen et al 1994).

Meanwhile, one coactivator may be a target of several activators, creating additional synergistic actions (Sauer et al 1997).

Different And Common Features Of CCR4 And ADR1

CCR4 is required for cell wall integrity and has a leucine-rich repeat motif that is required for protein-protein interactions. In contrast, ADR1, which has a zinc-finger motif and binds DNA directly, is required for peroxisome genes and genes involved in fatty acid synthesis. As shown in Figure 20, these two proteins share several common features. First, both are required for full expression of the ADH2 gene. Second, their protein levels are both elevated significantly on ethanol compared with those on glucose medium. Third, ADR1 and SNF1 both are required for derepression of glucose while CCR4 suppress snf1 and other components in the CCR4-NOT may mediate the interaction of CCR4 with SRB/SSN mediator, which also suppresses the phenotypes of snf1 (refer to introduction, Chapter1). Fourth, both interact with TFIID and SAGA directly or indirectly. NOT2 in the CCR4-NOT complex interacts with GCN5 genetically and NOT1 associates with TBP/SPT15 biochemically (Kuras et al 1998, Badarinarayana et al 2000, Lee et al 1998). ADR1 physically interacts withs GCN5 (Chiang et al 1996) and also exhibited multiple interactions with TFIID (Kormanitsky et al 1998, Bai in prep).

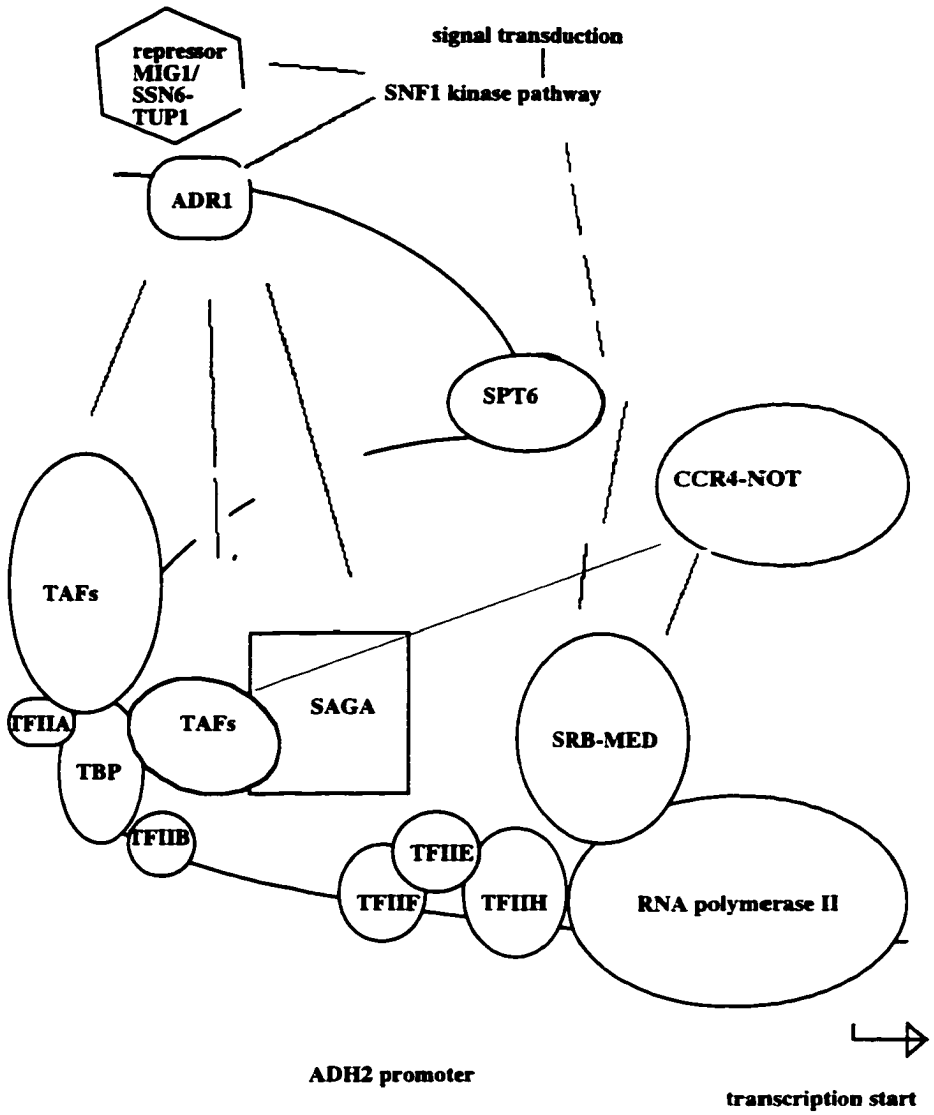

Figure 20. Model of the CCR4-NOT complex and ADR1 in the transcriptional activation at ADH2 gene. This drawing represents one of the current model on activated transcription as well as the results of numerous studies on ADR1. Transcriptional activator (s) bind(s) upstream activating sequence and induce(s) or stabilize(s) an active preinitiation complex (PIC) through interacting with multiple components in the PIC.

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APPENDIX

Dhh1p, a Putative RNA Helicase, Associates with the General Transcription Factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*

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ABSTRACT

The POP2 (Caf1) protein in *Saccharomyces cerevisiae* affects a variety of transcriptional processes and is a component of the Ccr4p complex. We have isolated five multicopy suppressor genes of a *pop2* deletion mutation: *CCR4*, *DHH1* (a putative RNA helicase), *PKC1*, *STM1*, and *MPT5* (*m*ulticopy suppressor of *p*op *t*wo). Overexpression of either the *CCR4* or *DHH1* genes effectively suppressed phenotypes associated with *pop2* mutant cells; overexpression of *PKC1*, *STM1*, or *MPT5* genes produced only partial suppression. Disruption of the *CCR4* or *DHH1* genes resulted in phenotypes similar to those observed for *pop2* cells. In addition, overexpression of the *DHH1* gene also suppressed the *ccr4* mutation, suggesting a close relationship between the *POP2*, *CCR4*, and *DHH1* genes. Two-hybrid analysis and coimmunoprecipitation experiments revealed that Pop2p and Dhh1p interact physically, and these and other data suggest that Dhh1p is also a component of the Ccr4p complex. Finally, we investigated the genetic interaction between factors associated with *POP2* and the *PKC1* pathway. The temperature-sensitive growth defect of *dhh1* or *mpt5* cells was suppressed by overexpression of *PKC1*, and the defect of *mpk1* cells was suppressed by overexpression of *MPT5*. These results and phenotypic analysis of double mutants from the *POP2* and *PKC1* pathways suggested that the *POP2* and the *PKC1* pathways are independent but have some overlapping functions.

THE *POP2* (*CAF1*) gene is required for glucose derepression of gene expression in *Saccharomyces cerevisiae* (Sakai *et al.* 1992; Draper *et al.* 1995). The *pop2* mutant cells exhibit many defects, including reduced levels of reserve carbohydrates, resistance to glucose derepression, temperature sensitivity for growth, increased *PGK1* transcription during stationary phase, and reduced levels of alcohol dehydrogenase II, isocitrate lyase, and invertase (Sakai *et al.* 1992; Draper *et al.* 1995). Pop2p has been shown to be part of the Ccr4p complex (Draper *et al.* 1995; Liu *et al.* 1997). Ccr4p, a general transcription factor, is required for the transcription of many genes, including glucose-repressible *ADH2*, and the pleiotropic nature of defects in *CCR4* is similar to that in *POP2* (Denis and Malvar 1990; Malvar *et al.* 1992; Sakai *et al.* 1992). Homologs of the *POP2* gene have been identified from humans, mice, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (Draper *et al.* 1995). The high degree of evolutionary conservation and their functional interchangeability suggest that the *POP2* gene plays an important functional role in cells.

We have identified several new phenotypes of *pop2* mutants. First, *pop2* cells are sensitive to staurosporine, a potent inhibitor of protein kinase C that is encoded by *PKC1*. Second, *pop2* cells are also sensitive to caffeine (Liu *et al.* 1997). In addition, the temperature-sensitive phenotype of *pop2* is suppressed by the addition of 1 M sorbitol to the medium. These phenotypes are characteristic of mutants involved in the *PKC1-MPK1* pathway (Thevelein 1994), which controls cell wall integrity. Cells carrying *pkc1*Δ or *mpk1*Δ cannot use glycerol as a sole carbon source (Costigan *et al.* 1994), and this suggests the involvement of the *PKC1-MPK1* pathway in the transcription of genes required for glycerol utilization. To understand the functions of Pop2p, we have searched for multicopy suppressor genes of a *pop2* deletion mutation. This approach has proved to be quite useful for analyzing many cellular processes such as the *SNF1* kinase pathway (Estruch and Carlson 1993; Hubbard *et al.* 1994) and the *PKC1-MPK1* protein kinase pathway (Lee *et al.* 1993a; Nickas and Yaffe 1996). Using this approach, we hoped to obtain genes encoding proteins with functional relationships to *POP2*: negative regulators of an antagonistic pathway or positive regulators of

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TABLE 1

List of yeast strains

Strain	Genotype	Source
D40	*MATa/MATα ade8/ade8 aro7/ARO7 his3/his3 leu2/leu2 TRP1/trp1 ura3/ura3*	This work
A475	*MATa can1 gal2 leu2 met14 trp1 ura3 rgr1Δ2::URA3*	SAKAI, 1990
A880	*MATa aro7 his3 leu2 ura3 pop2-Δ3::URA3*	This work
A1123	*MATα leu2 met14 trp1 ura3*	This work
A1152	*MATα aro7 his3 leu2 tmt14 trp1 ura3 stl1-1 (pkc1^{ts})*	This work
A1158	*MATa aro7 his3 leu2 trp1 ura3 pkc1Δ::HIS3*	This work
A1385	*MATα ade8 his3 leu2 trp1 ura3 dhh1Δ::URA3*	This work
A1413	*MATa ade8 his3 leu2 trp1 ura3 mpt5Δ::HIS3*	This work
A1454	*MATa aro7 ade8 his3 leu2 ura3 stm1Δ::ADE8*	This work
A1522	*MATα aro7 ade8 his3 leu2 trp1 ura3 ccr4Δ::HIS3*	This work
612-1d	*MATa adh1-11his3 leu2 trp1 ura3*	This work
612-1d-d1	*MATa adh1-11 his3 trp1 leu2 ura3 dhh1Δ::URA3*	This work
EGY188	*MATa his3 leu2 trp1 ura3 LexAop-LEU2*	This work
EGY191	*MATα his3 leu2 trp1 ura3 LexAop-LEU2*	This work
L40	*MATa his3 leu2 trp1 ura3 LYS::LexAop-HIS3 URA3::LexAop-lacZ*	T. TANAKA, Shimane University
DL251	*MATa/MATα can1/can1 his4/his4 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-52/ura3-52 bck1Δ::URA3/bck1Δ::URA3*	D. L. LEVIN, Johns Hopkins University
DL456	*MATa/MATα can1/can1 his4/his4 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-52/ura3-52 mpk1Δ::TRP1-/mpk1Δ::TRP1*	D. L. LEVIN
KAN128	*MATa can1 his4 leu2-3,112 trp1-1 ura3-52 mkk1Δ::LUE2 mkk2Δ::URA3*	K. IRIE, Nagoya University

a downstream pathway. We report here the isolation of five multicopy suppressor genes of the *pop2* deletion mutation in *S. cerevisiae*. We also present genetic and biochemical evidence indicating that Pop2p, Ccr4p, and Dhh1p are part of the same complex of proteins. A possible interaction between the *POP2* and *PKC1-MPK1* pathways is discussed.

MATERIALS AND METHODS

Strains and genetic methods: The strains of *S. cerevisiae* used in this study are listed in Table 1. Crossing, sporulation, and tetrad analyses were carried out by standard genetic methods (SHERMAN *et al.* 1986). Unless otherwise specified, the permissive and restrictive temperatures were 24° and 36°, respectively. The transformation of yeast was performed by the LiOAc method (ITO *et al.* 1983). *Escherichia coli* strains HB101 and JM109 were used as hosts for constructing and propagating plasmids. The transformation of *E. coli* was performed as described (HANAHAN 1983). The basic culture medium used for *S. cerevisiae* was YPD medium containing 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose (SHERMAN *et al.* 1986). The synthetic medium was CSM medium containing 0.67% yeast nitrogen base without amino acids, 2% dextrose, and amino acids as required (SHERMAN *et al.* 1986). The media were solidified with 2% Bacto-agar for plates. To test the sensitivity of various mutants to staurosporine (Kyowa Medix, Tokyo, Japan), the drug was dissolved in dimethyl sulfoxide and added to YPD media to a final concentration of up to 5 μg/ml (TAMAOKI *et al.* 1986; YOSHIDA *et al.* 1992). Tetrad dissection of heterozygous diploids carrying *pkc1Δ* was performed on a YPD plate containing 1 M sorbitol. Luria broth was supplemented with ampicillin for selection of the *E. coli* transformants as described (MANIATIS *et al.* 1982).

Preparation of DNA and RNA: Preparation of *E. coli* DNA, Southern hybridization, and Northern hybridization were performed as described (MANIATIS *et al.* 1982). The preparation of yeast DNAs and Northern analysis were performed as described (SHERMAN *et al.* 1986).

ADH II assay: Yeast cells were grown in YEP medium supplemented with either 8% glucose or 3% ethanol, and the activity of ADH II was measured as described previously (COOK *et al.* 1994).

Isolation of multicopy suppressors of *pop2-Δ4*: A yeast genomic DNA library (in YEp13, *LEU2* marker) was transformed to an A880 strain carrying the *pop2-Δ4* mutation (SAKAI *et al.* 1992), and transformants were selected on CSM lacking leucine at 24° for 5 days. To isolate suppressors for the temperature-sensitive phenotype of *pop2* cells, transformants were replica plated on YPD, and the plates were further incubated at 36°. To isolate suppressors for the staurosporine-sensitive phenotype of *pop2* cells, the transformants were replica plated on YPD containing 1 μg/ml staurosporine. Of 25,000 transformants, 85 colonies were selected. The 85 suppressor plasmids were recovered, analyzed by Southern hybridization and restriction mapping, and divided into six classes. The largest class (containing 65 plasmids) was identified as *POP2* itself. The remaining five classes were tentatively called MPTs (*m*ulticopy suppressor of *p*op *t*wo). The classes of *MPT1*, *MPT2*, *MPT3*, *MPT4*, and *MPT5* contained 13, 3, 2, 1, and 1 plasmids, respectively. To localize the suppressor activities for these plasmids, individual restriction fragments were subcloned into the YEp213 vector and tested for their abilities to grow on YPD plates at the restriction temperature or YPD plates containing staurosporine. These *MPT* genes were further subcloned into pUC18, and the nucleotide sequences were determined by the Sanger method using a Sequenase Kit (United States Biochemical, Cleveland, OH). Nucleotide sequence analysis revealed that three genes had been identified previously. *MPT1* is *DHH1*, which encodes a putative RNA helicase (STRAHL-BOLSINGER and TANNER 1993), *MPT2* is identical to *CCR4*, which is required for the full expression of

the glucose-repressible *ADH2* gene (Denis and Malvar 1990; Malvar *et al.* 1992), and *MPT3* turned out to be *PKC1*, a yeast homolog of mammalian protein kinase C (Levin *et al.* 1990). *MPT4* is *STM1*, a guanine quartet–binding protein (Frantz and Gilbert 1995). *MPT5* is identical to *HTR1*. The name *HTR1* had been used for a different gene (Özcan *et al.* 1993); we refer to this gene as *MPT5*.

Disruption of *DHH1*, *CCR4*, *PKC1*, *MPT4*, and *MPT5*: The *dhh1Δ::URA3*, *ccr4Δ::HIS3*, *pkc1Δ::HIS3*, *stm1Δ::ADE8*, and *mpt5Δ::HIS3* alleles were constructed by the standard method and transformed into diploid (D40) cells to replace the chromosomal loci by the one-step gene disruption method (Rothstein 1983).

Construction of dhh1Δ::URA3: A 2.5-kbp DNA fragment (*Pma*CI-*Apa*LI) bearing the *DHH1* gene (ORF: 1518 bp) was filled in with Klenow enzyme and subcloned into the *Sma*I site of pBluescript II. The resulting plasmid DNA was digested with *Bst*EII and *Bgl*II. This step deleted 115 bp within the coding region (+438 to + 552). After the ends were filled in with Klenow enzyme, a 1.2-kbp *URA3* fragment, isolated by digestion of YEp24 plasmid DNA with *Hin*dIII and filled in, was inserted. The resulting plasmid was digested with *Pvu*I and *Kpn*I and used for transformation. After heterozygous disruption was confirmed by Southern blotting, the transformants were subjected to sporulation followed by micromanipulation to generate haploid cells carrying the *dhh1* deletion mutation.

Construction of ccr4Δ::HIS3: The *ccr4Δ::HIS3* allele was introduced in our background and L40 strain using the TM1 plasmid as described (Malvar *et al.* 1992).

Construction of pkc1Δ::HIS3: A 4.3-kbp *Sph*I-*Sph*I fragment bearing the *PKC1* gene was recovered from the original *MPT3* plasmid. Disruption of the *PKC1* gene was performed by the replacement of a 0.6-kbp *Bam*HI fragment of the *PKC1* gene with the *HIS3* gene from pJJ216 as described (Yoshida *et al.* 1992).

Construction of stm1Δ::ADE8: A 1600-bp DNA fragment (*Pma*CI-*Cla*I) bearing the *MPT4* gene (ORF: 819 bp) was filled in with Klenow enzyme and subcloned into the *Sma*I site of the pUC18 plasmid whose *Pst*I site was disrupted. The resulting plasmid was digested with *Xba*I and *Hpa*I to remove 33 bp within the *MPT4* gene (+283 to +316) and a 1152 bp *ADE8* DNA whose ends were *Xba*I and *Sma*I. The resulting plasmid was digested with *Eco*RV and *Afl*II, and was used for transformation.

Construction of mpt5Δ::HIS3: A 5-kbp *Aat*II-*Aat*II DNA fragment bearing the *MPT5* gene was isolated from the original *MPT5* plasmid DNA, whose ends were changed to *Sal*I by linker ligation, and subcloned into the *Sal*I sited of pUC18. The plasmid DNA was digested with *Nsp*V to remove −420 to +3700 of the *MPT5* DNA. After the ends were filled in with Klenow enzyme, a 1.8-kbp *HIS3* DNA whose ends were filled in with Klenow enzyme was inserted. The resulting plasmid was digested with *Eco*RI and *Eco*RV, and was used for transformation.

All disruptions were introduced into D40 cells, and the disruptions were confirmed by Southern blot analysis. Haploid cells carrying the disruption were recovered by standard microdissection.

Two-hybrid analysis: Plasmid pBTM116 (kindly provided by Katsunori Tanaka, Shimane University) was used to construct LexA-Pop2p. *Bam*HI sites were introduced into the DNA encoding the *POP2* protein by PCR, and the resulting DNA fragment was subcloned into the *Bam*HI site of pBMT116 in-frame to LexA. Plasmid pGADGH was purchased from Clontech (Palo Alto, CA) and was used to construct Gal4 activation domain fusion plasmids (Gal4AD-Ccr4 and Gal4AD-Dhh1). Yeast strain L40 was used as the two-hybrid host (Table 1). The activity of β-galacto-sidase was determined as described (Miller 1972). The LexA-Vpu plasmid contains the hydrophilic segment of Vpu (residues 33–81) fused to full-length LexA (Chiang *et al.* 1996). B42-Pop2p contains residues 149–441 of Pop2p fused in frame to the *E. coli*–derived B42 transcriptional activator (Zervos *et al.* 1993). This segment of Pop2p interacts with Ccr4p in the two-hybrid assay and coimmunoprecipitates with Ccr4p (Draper *et al.* 1995; data not shown). B42-Sip1p contains residues 243–500 of Sip1p (Yang *et al.* 1992) fused to B42. LexA-Dhh1 was constructed by removing the complete *DHH1* sequence from the Gal4AD-Dhh1AD fusion using *Bam*HI and *Sal*I and cloning into the *Bam*HII and *Sal*I sites of LexA-202-1 (Cook *et al.* 1994).

Immunoprecipitation: Preparation of protein extracts and immunoprecipitation were performed as described (Draper *et al.* 1995). The polyclonal antibody against the Gal4 activa-

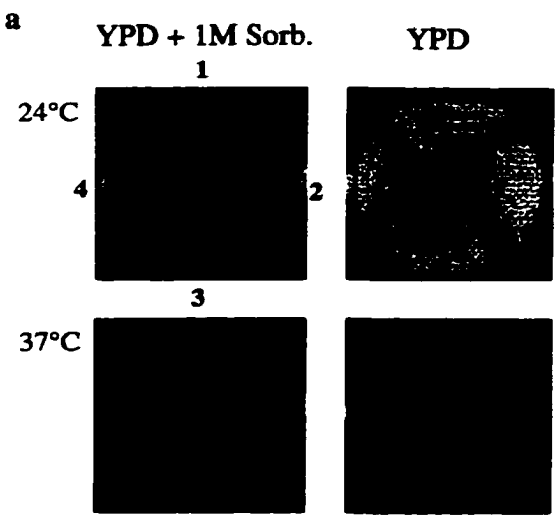


Figure 1.—Newly found phenotypes of *pop2* cells. (a) Sorbitol-suppressible, temperature-sensitive growth defect of *pop2* deletion cells. (1) A1123 (wild-type), (2) A1152 (*stt1-1/pkc1*^{ts}), (3) A475 (*ngr1-Δ2::URA3*), (4) A880 (*pop2-Δ4::URA3*). Cells were streaked on YPD or YPD+1 M sorbitol plates and were incubated at 24° and 37° for 4 days. (b) Staurosporine sensitivity. A1123 (wild-type) and A880 (*pop2-Δ4::URA3*) cells were resuspended with YPD media, spread on the YPD plate containing staurosporine, and incubated at 24° for 4 days.

TABLE 2

Phenotypes of the deletion mutants

	YPD			YPD + sorbitol		YPG		YPG + sorbitol			
	15°	24°	37°	24°	37°	24°	37°	24°	37°	Sta.	Caf.
pop2	−	+	−	+	+	±[a]	−	−	−	−	−
ccr4	−	+	−	+	+	±[a]	−	−	−	−	−
dhh1	−	+	−	+	+	±[a]	−	−	−	−	−
pkc1	−	−	−	+	−	−	−	−	−	−	−
stm1	+	+	+	+	+	+	−	+	−	+	+
mpt5	−	+	−	+	+	+	−	+	−	+	−

Exponentially growing cells were resuspended in water, and aliquots were spotted on the plate indicated. Plates were incubated at each temperature for 4 days. Plus signs indicate growing (formed colony); minus signs indicate not growing (not formed colony). Sta., 2 μg/ml staurosporine in YPD plate; Caf., 8 mM caffeine in a YPD plate.

[a] These mutants grew slowly on a YPGlycerol, but they did not grow on a CSMGlycerol plate. This phenotype varied depending on genetic background.

tion domain was a kind gift from KOUICHI ISHIGURO, Mitsubishi Kasei Institute of Life Sciences. The Ccr4p antibody was described previously (DRAPER *et al.* 1995). Antibodies against the HA1 epitope were commercially obtained. The sequence data presented in this paper have been submitted to the GenBank Data Libraries under the following accession numbers: *MPT4*, D26183; *MPT5*, D26184.

RESULTS

Isolation of multicopy suppressors of *pop2*: The temperature-sensitive phenotype of *pop2* mutants suppressed by the addition of 1 M sorbitol in the medium (Figure 1a). At the restrictive temperature, the *pop2* cells become enlarged and swollen, showing a cell lysis phenotype (data not shown). Furthermore, the *pop2* cells are also sensitive to staurosporine (1 μg/ml; Figure 1b), a potent inhibitor of the yeast protein kinase C homolog (*PKC1*; TAMAOKI *et al.* 1986; LEVIN *et al.* 1990; YOSHIDA *et al.* 1992). Finally, the growth of *pop2* cells is inhibited by low levels of caffeine (8 mM; Table 2). *pop2* cells also exhibited a weak cold-sensitive growth phenotype that is suppressible by 1 M sorbitol (LIU *et al.* 1997). These phenotypes are similar to those caused by mutations in the *PKC1* pathway, and they suggest that *POP2* plays a role in maintenance of cell wall integrity in addition to its other known phenotypic defects. Since the *pop2* mutant affects such varied processes, it was of interest to us to know the multifunctional roles of Pop2p in cells.

We searched for multicopy suppressors of the temperature- and staurosporine-sensitive growth defects of *pop2* mutation (see MATERIALS AND METHODS). We identified five gene products that suppressed the *pop2* phenotypes either completely or partially (Table 3). Nucleotide sequence analysis revealed that these genes are *DHH1*, which encodes a putative RNA helicase (STRAHL-BOLSINGER and TANNER 1993), *CCR4*, which is required for the full expression of the glucose-repressible *ADH2* gene (DENIS and MALVAR 1990; MALVAR *et al.* 1992), *PKC1*, a yeast homolog of mammalian protein kinase C (LEVIN *et al.* 1990), *STM1*, a guanine quartet–binding protein (FRANTZ and GILBERT 1995), and *MPT5* (KIKUCHI *et al.* 1994; KENNEDY *et al.* 1997). Mpt5p contains eight tandem copies of an ~38 amino acid–repeat, including a strong consensus sequence of 13 amino acids (LxxDxFGxxFLQK). This repeat is also found in YGL023 (CHEN *et al.* 1991) and in the Drosophila pumilio gene (BARKER *et al.* 1992; MACDONALD 1992). The biological function of this region remains to be identified.

Overexpression of either *CCR4* or *DHH1* suppressed all the *pop2* phenotypes. In contrast, overexpression of *MPT5*, *PKC1*, and *STM* only suppressed some of these

TABLE 3

Summary of multicopy suppression

Mutation		Gene on YEp213					
		POP2	*CCR4*	*DHH1*	*PKC1*	*STM1*	*MPT5*
pop2	ts	+	+	+	−	−	+
	sta	+	+	+	+	+	−
	gly	+	+	+	+	−	+
ccr4	ts	−	+	+	−	−	+
	sta	−	+	+	−	+	+
dhh1	ts	−	−	+	+	−	−
	sta	−	−	+	+	−	−
pkc1	ts	−	−	−	+	−/+	−
	sta	−	−	−	+	+	−
mpt5	ts	−	−	−	+	−	+
	caf	−	−	−	+	+	+

Transformants were suspended in water and spotted on YP plates containing either 2% glucose, 2% glucose + 2 μg/ml staurosporine, 2% glucose + 8 mM caffeine, or 2% glycerol. The plates were incubated at 24° or 37° for 4 days. ts, incubated at 37°; sta, 2 μg/ml staurosporine; caf, 8 mM caffeine; gly, 2% glucose.

phenotypes (Table 3). These results suggested a close genetic relationship among *POP2*, *CCR4*, and *DHH1*, while the interaction between *POP2* and *PKC1*, *STM1*, or *MPT5* might be relatively weak.

Northern analysis: It is possible that the overexpression of these genes in *pop2* cells suppressed the phenotypic defects if their transcription were being positively regulated by the Pop2. To examine this possibility, we measured the mRNA levels of each suppressor gene in wild-type and *pop2* deletion cells. No significant differences in the mRNA amounts were observed between wild-type and *pop2* deletion cells for any of the suppressor genes (data not shown).

Genetic interactions among *POP2*, *CCR4*, and *DHH1*: Disruptions of *POP2*, *CCR4*, and *DHH1* were constructed (see MATERIALS AND METHODS), and the resulting phenotypes were examined. In addition to the phenotypes previously observed for a *CCR4* disruption (MALVAR *et al.* 1992; DRAPER *et al.* 1995; Table 2), we found that in strain backgrounds other than those of the originally reported, *ccr4*Δ cells are also temperature sensitive for growth, which is suppressed by addition of 1 M sorbitol to the medium. Furthermore, the *ccr4*Δ cells are sensitive to staurosporine and caffeine. While it has been reported that the disruption of *DHH1* does not cause any phenotype (STRAHL-BOLSINGER and TANNER 1993), we found that *dhh1*Δ cells in our genetic background show a temperature-sensitive cell lysis phenotype that is suppressed by 1 M sorbitol (Table 2). Proteins with RNA helicase activity are often involved in mRNA transport or RNA processing (MARGOSSIAN and BUTOW 1996; DALBADIE-MCFARLAND and ABELSON 1990). To examine *dhh1* effects on mRNA transport, *dhh1* cells were shifted to 37° for 1 hr, and mRNA accumulation in the nucleus was measured by in situ hybridization with an oligo dT probe. No significant difference was detected between *dhh1* and wild-type cells (data not shown). We also failed to detect pre-mRNA accumulation of the intron-containing *CYH2* gene in *dhh1* cells (T. TANI, personal communication). These observations suggest that the *dhh1* mutation did not affect RNA processing. Cells carrying the *dhh1* mutation also showed cold-sensitive growth at 15°, do not grow on a CSMGlycerol plate, and grow slowly on a YPGlycerol plate. Furthermore, *dhh1*Δ cells are sensitive to staurosporine and caffeine (Table 2, data not shown). Table 4 also shows that *DHH1* is required for the full expression of the *ADH2* gene, a phenotype previously observed with *ccr4* (DENIS 1984) or *pop2/caf1*–deleted cells (DRAPER *et al.* 1995). These phenotypic analyses confirm a close functional link between *POP2*, *CCR4*, and *DHH1*. In contrast, disruption of *PKC1*, *STM1*, and *MPT5* displays only some phenotypic similarities to *pop2*, *ccr4*, and *dhh1* (Table 2) and their mutant phenotypes were consistent with those observed previously (LEVIN and BARTLETT-HEUBSH 1992; FRANTZ and GILBERT 1995; KIKUCHI *et al.* 1994; CHEN and KURJAN 1997). It is noteworthy that temperature-sensitive growth of *mpt5* cells was suppressed by 1 M sorbitol.

TABLE 4

***DHH1* is required for ADH II expression**

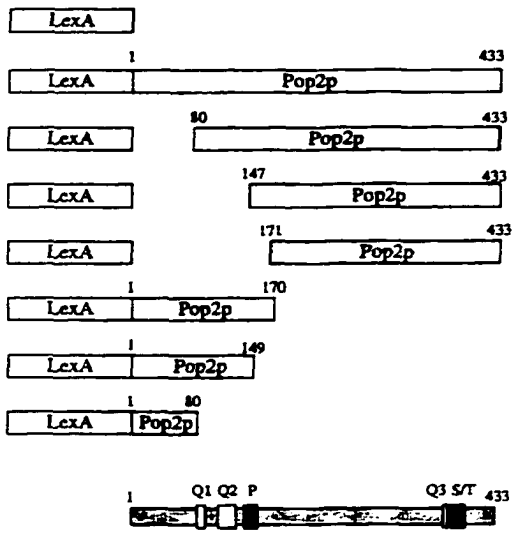
	ADH II activity (mU/mg)	
Genotype	Glucose	Ethanol
wt	<5	2700
dhh1	<5	550

Wild-type (612-1d) or *ddh1* (612-1d-d1) cells were used. Activity of ADH II was determined as described in MATERIALS AND METHODS. Standard errors are <15%.

Multicopy plasmids carrying *POP2*, *CCR4*, and *DHH1* were introduced into each mutant in different combinations, and the effects on their growth defects were tested. The *pop2* mutation was suppressed by *CCR4* or *DHH1*, and the *ccr4* mutation was also suppressed by *DHH1* (Table 3); however, the growth defects in *dhh1* cells were not suppressed by either *POP2* or *CCR4*. To further test the epistasis among these genes, cells carrying double mutations were constructed and the phenotypic additivity was tested. We did not find any additive phenotypes in the double-mutant cells, suggesting that *POP2*, *CCR4*, and *DHH1* function in the same pathway, and that *CCR4* and *DHH1* genetically function downstream of *POP2*.

Dhh1p physically interacts with Pop2p: The physical association of Dhh1p with Pop2p and Ccr4p was analyzed by two-hybrid analysis and coimmunoprecipitation experiments. The combination of LexA-Pop2 and Gal4AD-Dhh1AD in the two-hybrid system resulted in a significant increase in β-galactosidase activity beyond that obtained with LexA-Pop2p alone (Figure 2, Table 5). LexA-Dhh1p also was observed to interact specifically with a B42-Pop2 fusion, resulting in 18 U/mg of β-galactosidase activity as compared to 2.5 U/mg activity for the interaction of LexA-Dhh1 with B42 alone. Figure 2 shows that residues 147–171 of Pop2p are necessary for the interaction. This region is similar to that which was observed to be necessary for the Pop2p and Ccr4p interaction (DRAPER *et al.* 1995). Also, disruption of *CCR4* did not abrogate the interaction between LexA-Pop2 and Gal4AD-Dhh1 (Table 5). It should be noted that a *ccr4* disruption reduced the transcriptional activity of LexA-Pop2, which is a general effect that *ccr4* has on LexA activators (DRAPER *et al.* 1995). We further tested the possibility of an interaction in the two-hybrid system between LexA-Ccr4 and Gal4AD-Dhh1 or B42-Dhh1, and between LexA-Dhh1 and B42-Ccr4, but we were unable to detect any interaction between Ccr4p and Dhh1p, although each of these proteins was expressed in yeast (data not shown). These results suggest that Ccr4p and Dhh1p do not interact directly.

To confirm the physical interaction between Pop2p and Dhh1p, a coimmunoprecipitation experiment was



	β-Gal (U/mg protein)		
	Gal4AD	Gal4AD-Ccr4	Gal4AD-Dhh1
LexA	26	27	66
LexA-Pop2p (1–433)	430	4,400	1,700
LexA-Pop2p (80–433)	350	3,400	1,100
LexA-Pop2p (147–433)	91	2,080	520
LexA-Pop2p (171–433)	5	5	5
LexA-Pop2p (1–170)	5	4	4
LexA-Pop2p (1–149)	6	8	11
LexA-Pop2p (1–80)	4	8	7

FIGURE 2.—Region of Pop2p responsible for the interaction with Dhh1p. β-Galactosidase assays and interactions with Gal4p-Dhh1p were conducted as described in MATERIALS AND METHODS. Amino acid residues of each segment of Pop2p are indicated. All assays and interactions were done in strain L40. All LexA-Pop2p derivatives were expressed at equivalent levels as assessed by Western analysis (data not shown). Schematic illustration shows location of the characteristic domains. Q1, polyglutamine stretch (residues 81–91); Q2, polyglutamine stretch (residues 112–126); Q3, glutamine-rich region (residues 364–371); P, proline-rich region (residues 139–155); S/T, serine/threonine-rich region (residues 375–391). All the values were the average of at least five independent experiments. Standard errors were <15%.

carried out using transformants carrying LexA-Dhh1 and B42-Pop2 that was tagged with the HA1 epitope (designated HA1-Pop2 in Figure 3). Immunoprecipitating LexA-Dhh1 with anti-LexA antibody resulted in the coimmunoprecipitation of B42-Pop2 (Figure 3, lane 5). The presence of the B42-Pop2 fusion in the immunoprecipitated materials was dependent on LexA-Dhh1 (Figure 3, lane 6). Sip1p, a protein (YANG *et al.* 1992) that is not part of the Ccr4p complex (LIU *et al.* 1997), did not coimmunoprecipitate with the LexA-Dhh1 fusion (Figure 3, lane 4). These results suggest that Dhh1p is physically associated with the CCR4 complex, and that Pop2p can physically interact with both Ccr4p and Dhh1p.

Genetic interaction between *POP2* and *PKC1-MPK1* pathways: Because *PKC1* was isolated as a weak multicopy suppressor of the *pop2* mutation, and the phenotypes of *pop2* cells and *mpt* cells are similar to cells carrying mutations involved in the *PKC1-MPK1* pathway, we determined the epistatic relationship among these genes. Overexpression of *POP2*, *CCR4*, or *DHH1* in *pkc1Δ*, *bck1Δ*, *mkk1Δ* *mkk2Δ*, and *mpk1Δ* mutant cells did not suppress any of their phenotypes, including temperature-sensitive growth and caffeine sensitivity. *PKC1* suppressed

TABLE 5

Pop2p interacts with both Ccr4p and Dhh1p in the two-hybrid system

Host	DNA-binding hybrid	β-Galactosidase activity (U/mg protein) Activation hybrid		
		Gal4AD	Gal4AD-Ccr4	Gal4AD-Dhh1
L40	LexA	25	27	61
	LexA-Pop2	430 (1.0)	4,400 (10.2)	1,700 (4.0)
L40/*ccr4*	LexA	0.7	2.4	1.2
	LexA-Pop2	20 (1.0)	660 (31)	67 (3.2)

Transformants were grown in selective CSM medium. Values are averages of assays of three to five different transformants. Standard errors were <15%. Parentheses indicate fold of induction.

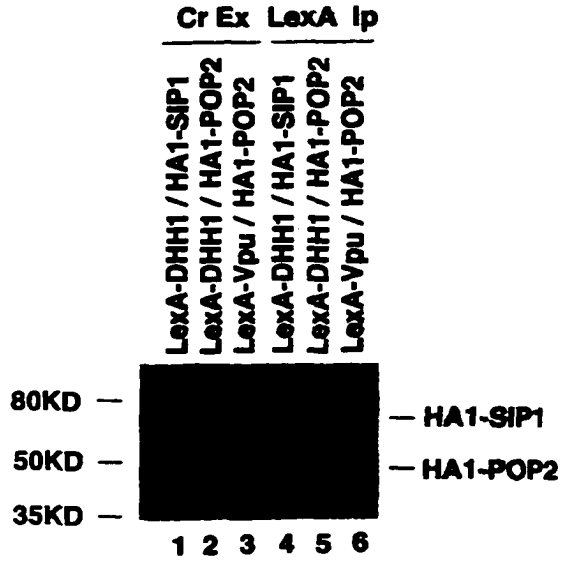


FIGURE 3.—*LexA-Dhh1p coimmunoprecipitates with* Pop2p. Crude extracts (lanes 1–3) were prepared from diploid EGY188/EGY191 containing the LexA and B42 (HA1) fusion proteins as indicated. Immunoprecipitations (lanes 4–6) were conducted from these extracts using an antibody directed against LexA. Extracts and immunoprecipitated samples were separated by SDS-PAGE, and proteins were identified by using an antibody directed against the HA1 epitope. LexA-Dhh1p and LexA-Vpu were expressed to comparable extents (data not shown). The protein that comigrates with HA1-Pop2p in lane 1 is a degradation product of HA1-Sip1p and does not coimmunoprecipitate with LexA-Dhh1p (see lane 4).

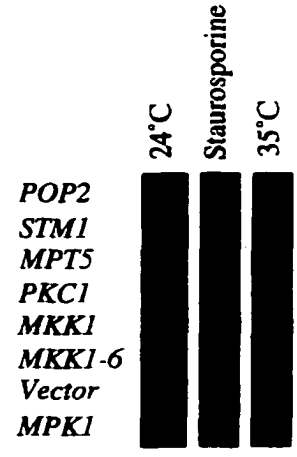


FIGURE 4.—*PKC1* and *MPT5* suppressed the *mpk1* mutation. Homozygous diploid cells carrying the *mpk1* deletion mutation (DL456) were transformed with YEp213 bearing various genes indicated. The transformants were suspended in water and spotted on YPD plates and a YPD plate containing 2 μg/ml staurosporine. The plates were incubated at 24° or 35° for 4 days. *MKK1-6* encodes a dominant active form of Mkk1p kinase (LEE *et al.* 1993b).

the temperature-sensitive growth of *mpk1* deletion cells at 35° (Figure 4). Overexpression of *STM1* suppressed the staurosporine- and caffeine-sensitive growth, not only of *pop2Δ* mutants, but also of *pkc1Δ*, *bck1Δ*, *mkk1Δ* *mkk2Δ*, or *mpk1Δ* mutants. Thus, *STM1* may function downstream of these genes. *MPT5* overexpression suppressed temperature-sensitive growth defect of *mpk1Δ* deletion cells at 35° (Figure 4), but no other phenotype of this mutant. These results suggest an interaction between the *POP2* and *PKC1-MPK1* pathways, and that Ccr4 and Dhh1 may function downstream of both the *POP2* and *PKC1-MPK1* pathways.

POP2 and *PKC1* pathways may function independently but have overlapping functions. To examine these possibilities, double-mutants carrying *pop2Δ dhh1Δ*, *pop2Δ* *mpt5Δ*, and *dhh1Δ mpt5Δ* were constructed. They all showed temperature-sensitive phenotypes. No phenotypic additivity was observed, except that the temperature-sensitive growth of *dhh1Δ mpt5Δ* double-mutant cells was not suppressed by 1 M sorbitol (data not shown). On the other hand, haploid cells carrying *pkc1Δ pop2Δ*, *pkc1Δ dhh1Δ*, or *pkc1Δ mpt5Δ* were not recovered. Among the tetrads analyzed (10 for *pop2Δ* and *pkc1Δ*, eight for *dhh1Δ* and *pkc1Δ*, and eight for *mpt5Δ* and *pkc1Δ*), most tetrads contained less than three viable spores, even on the YPD plate containing 1 M sorbitol (data not shown). Judging from the disruption markers, all the dead cells were found to carry either *pop2Δ pkc1Δ*, *dhh1Δ pkc1Δ*, or *mpt5Δ pkc1Δ*. This synthetic lethality displayed phenotypic additivity and suggested that the *POP2* pathway and *PKC1* function independently. Taken together, the phenotypic similarity and genetic suppression among these mutants suggest that *POP2* and *PKC1* pathways function independently but have some overlapping functions.

DISCUSSION

Dhh1p, a putative RNA helicase, associates with Pop2p and Ccr4p physically and functionally: We identified five multicopy suppressor genes of the *pop2* deletion mutation. Two of these, *CCR4* and *DHH1*, appear functionally related to *POP2*. The overexpression of *DHH1* or *CCR4* rescues all the *pop2* defects tested, including high-temperature–sensitive growth, cold-sensitive growth, the inability to use glycerol, and staurosporine and caffeine sensitivity (Tables 2 and 3). Overexpression of *DHH1* also suppresses the growth defects in *ccr4* cells. In addition, *dhh1Δ* cells display a spectrum of phenotypes similar to *pop2Δ* or *ccr4Δ* cells. Importantly, *DHH1* was required for the full expression of the *ADH2* gene (Table 4), another phenotype shared by *pop2* and *ccr4* cells. Furthermore, cells carrying the double mutations *pop2Δ ccr4Δ*, *pop2Δ dhh1Δ*, or *ccr4Δ dhh1Δ* showed no phenotypic additivity (data not shown; DRAPER *et al.* 1995). These results suggest that there exists a close genetic interaction among the *POP2*, *CCR4*, and *DHH1* genes, and that these three proteins function together to control transcriptional processes. Because Dhh1p is

a putative RNA helicase but no defect in RNA processing was apparent in *dhh1* cells, we think that Dhh1p is involved in a novel aspect of gene expression.

Pop2p and Dhh1p were found to interact physically (Table 5, Figure 3), suggesting that Dhh1p is associated with the Ccr4p regulatory complex. Because we have no evidence to suggest an interaction between Ccr4p and Dhh1p by two-hybrid analysis, it cannot be excluded that Dhh1p interacts with Pop2p in a complex separate from that which associates with Ccr4p. This seems unlikely, however, since Dhh1p has also been found to coimmunoprecipitate with Dbf2p and Caf17p, two other Ccr4p complex components (unpublished observations; Liu *et al.* 1997). More importantly, nearly all the Ccr4p in the cells copurifed with Pop2p (Caf1p; M. Liu, personal communication), suggesting that there is no separate complex. Because there are multiple components of the Ccr4p complex, it is difficult at this point to clearly determine the order and directness of the interactions between these proteins.

Because overexpression of *PKC1*, *STM1*, and *MPT5* suppressed some phenotypes of *pop2Δ* cells, these genes might function further downstream of *POP2* or bypass some Pop2p functions (Table 3). Stm1p has G4 nucleic acid–binding activity (Frantz and Gilbert 1995), and a null allele of the *stm1* mutation does not cause phenotypes, as observed with the other *mpt* mutations. The relationship between the G4 nucleic acid–binding activity and *POP2* function remains to be elucidated. *MPT5* is required for growth at high temperatures and for the recovery from mating factor–induced G1 arrest (Kikuchi *et al.* 1994), and it is involved in yeast cell aging by redistribution of the Sir2p-Sir3p-Sir4p complex from the telomeres to the nucleolus (Kennedy *et al.* 1997). *MPT5* has been shown to interact with Sst2p and Cdc28p, which is involved in the pheromone signaling pathway and the cell cycle control pathway, respectively (Chen and Kurjan 1997). Since the *MPT5* gene is also involved in the stress response, it may be a general suppressor of temperature stress effects.

Since the *pop2Δ* and *mpt5Δ* showed no phenotypic additivity, *POP2* and *MPT5* also appear to function in the same pathway. Based on the facts that overexpression of *DHH1* or *MPT5* can suppress the *ccr4Δ* mutants, whereas overexpression of CCR4 had no effect on *dhh1Δ* or *mpt5Δ* cells (Table 3), we hypothesize that *DHH1* and *MPT5* function genetically downstream of *CCR4*. Overexpression of *DHH1*, however, could not suppress any phenotypes of *mpt5Δ* cells and vice versa. Furthermore, the *dhh1Δmpt5Δ* double-mutant cells showed temperature-sensitive growth, and this defect was not suppressed by 1 M sorbitol (data not shown). These results suggest that *DHH1* and *MPT5* genetically function in two independent pathways that act after *POP2* and *CCR4*.

Interaction between the *POP2* and *PKC1-MPK1* pathways: The protein kinase C pathway, including *PKC1*, *BCK1*, *MKK1* or *MKK2*, and *MPK1*, is important for cell wall integrity in yeast (Thevelein 1994). Mutants of *PKC1* require an osmotic stabilizer (*e.g.*, 1 M sorbitol) for survival. Also, cells carrying the *pkc1Δ* or *mpk1Δ* mutation cannot use glycerol as a sole carbon source (Costigan *et al.* 1994, Table 2). This may suggest that the *PKC1-MPK1* pathway activates the transcription of genes that require nonfermentable carbon sources and those that are involved in cell wall integrity. Since *PKC1* was isolated as a multicopy suppressor of *pop2Δ*, and most of the *mpt* mutants including *pop2* itself showed staurosporine-sensitive phenotypes and inability to utilize nonfermentable carbon sources, and the temperature-sensitive cell lysis phenotype of *pop2Δ* and the cold sensitivity phenotypes of *ccr4*, *pop2(caf1)*, and *dbf2*, were suppressed by 1 M sorbitol (Liu *et al.* 1997), it was of great interest to us to investigate the relationship between the *POP2* pathway and *PKC1* pathway.

Overexpression of *PKC1* suppressed temperature-sensitive growth of *dhh1Δ* or *mpt5Δ* mutants (Table 3). This suggests that *DHH1* and *MPT5* function upstream of *PKC1*. But cells carrying double mutations of *pop2Δpkc1Δ*, *dhh1Δ pkc1Δ*, or *mpt5Δ pkc1Δ* were synthetically lethal, ruling out a direct and dependent interaction among *PKC1* and *POP2*, *DHH1*, and *MPT5* (data not shown). In addition, the *dhh1Δ* and *mpt5Δ* mutations were suppressed by overproduction of *PKC1* but not by *MPK1* (data not shown), and overproduction of *MPT5* suppressed the *mpk1* mutation (Figure 4). Because epistasis among these genes is unclear, we speculate that the *POP2* pathway and the *PKC1* pathway are two independent pathways that have overlapping functions. To clarify this, we are currently searching for the common target of the two pathways. It should be noted that a well-accepted model of the *PKC1* pathway suggests that *MPK1* is epistatic to *PKC1* (Thevelein 1994). Our finding that overproduction of *PKC1* suppressed the *mpk1* mutation (Figure 4) seems to conflict with this model. It is possible that this might not be a direct effect, or that overproduction of *PKC1* might activate another pathway (Lee *et al.* 1993).

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The CCR4 and CAF1 Proteins of the CCR4-NOT Complex Are Physically and Functionally Separated from NOT2, NOT4, and NOT5†

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The CCR4-NOT complex (1 mDa in size), consisting of the proteins CCR4, CAF1, and NOT1 to NOT5, regulates gene expression both positively and negatively and is distinct from other large transcriptional complexes in *Saccharomyces cerevisiae* such as SNF/SWI, TFIID, SAGA, and RNA polymerase II holoenzyme. The physical and genetic interactions between the components of the CCR4-NOT complex were investigated in order to gain insight into how this complex affects the expression of diverse genes and processes. The CAF1 protein was found to be absolutely required for CCR4 association with the NOT proteins, and CCR4 and CAF1, in turn, physically interacted with NOT1 through its central amino acid region from positions 667 to 1152. The NOT3, NOT4, and NOT5 proteins had no significant effect on the association of CCR4, CAF1, and NOT1 with each other. In contrast, the NOT2, NOT4, and NOT5 interacted with the C-terminal region (residues 1490 to 2108) of NOT1 in which NOT2 and NOT5 physically associated in the absence of CAF1, NOT3, and NOT4. These and other data indicate that the physical ordering of these proteins in the complex is CCR4-CAF1-NOT1-(NOT2, NOT5), with NOT4 and NOT3 more peripheral to NOT2 and NOT5. The physical separation of CCR4 and CAF1 from other components of the CCR4-NOT complex correlated with genetic analysis indicating partially separate functions for these two groups of proteins. *ccr4* or *caf1* deletion suppressed the increased 3-aminotriazole resistance phenotype conferred by *not* mutations, resulted in opposite effects on gene expression as compared to several *not* mutations, and resulted in a number of synthetic phenotypes in combination with *not* mutations. These results define the CCR4-NOT complex as consisting of at least two physically and functionally separated groups of proteins.

The CCR4-NOT complex from *Saccharomyces cerevisiae* displays both positive and negative roles in the regulation of diverse genes and processes (6, 9, 20, 25). This complex, distinct from other large transcriptionally important complexes such as SNF/SWI, SAGA, SRB-containing polymerase II holoenzyme, and TFIID (10, 13, 20), consists of two forms, a 1.9×10^6-Da (1.9-mDa) and 1-mDa complex (20). The smaller complex consists of CCR4, CAF1 (POP2) (24), the five NOT proteins, and several unidentified proteins (20, 22, 23). Defects in components of this complex reduce expression of *ADH2* and other nonfermentative genes, affect the expression of genes involved in cell wall integrity, and suppress *spt10*-induced expression at the *ADH2* locus (9, 11, 13, 20, 22). Furthermore, mutations in *CCR4* or *CAF1* affect cell cycle progression in late mitosis (22). The *NOT* genes, in turn, were originally identified as repressing *HIS3* expression from a noncanonical TATA (TATA-less) element (5, 6), as well as affecting a number of other genes and processes (1, 8, 16). The recent demonstration that *not* and *caf1* mutations can suppress a defect in SRB4, a key component of the RNA polymerase II holoenzyme required for the transcription of most genes in yeast (19), further indicates a very general repressor role for the CCR4-NOT complex. It has been proposed that the NOT proteins inhibit transcriptional initiation by affecting TATA binding protein access to TATA-less sequences (4), a model in agreement with the fact that NOT1 has been found to associate with TATA binding protein (TBP) (19).

Of the proteins of the CCR4-NOT complex, only NOT1 is an essential protein (5). The C-terminal residues 1319 to 2108 of NOT1 are sufficient, however, for cells to remain viable (26). Pairwise combinations of *not* mutations do not in general lead to synthetic lethality (except for *not4* with *not5*), suggesting that they form a complex displaying overlapping functions (6, 23). However, CCR4 and CAF1 appear in certain contexts to be distinct from the other NOT proteins. Mutations in the five *NOT* genes result in increased resistance to 3-aminotriazole (3-AT) in a partially defective GCN4 background (6). This phenotype is not associated with CCR4 or CAF1 defects (20). Moreover, *not* mutations tend to increase *HO-lacZ* and *FKS1-lacZ* expression, whereas a *ccr4* or *caf1* deletion reduces expression or has little effect on these promoters (20). The CCR4 and CAF1 proteins also appear to be strongly associated; partial disruption of *CAF1* inhibits the association of CCR4 with the NOT1 and NOT2 proteins (20). Therefore, while *not* alleles have several phenotypes in common with *ccr4* and *caf1* defects (20), notably caffeine, temperature, and magnesium sensitivities, effects on *ADH2* and *CYC1* gene expression, and suppression of *spt10*-enhanced expression, CCR4 and CAF1 proteins may functionally and physically represent a separate group of proteins within the CCR4-NOT complex.

We have analyzed the association of CCR4, CAF1, and the NOT proteins and related these associations to the phenotypes of the constituents of this complex. The central segment of

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TABLE 1. Yeast strains used

Strain	Genotype
A790	*MATα aro7 his3 leu2 ura3*
A792	*MATα aro7 his3 leu2 ura3 pop2-Δ3::LEU2*
KY803	*MATa leu2-PET56 trp1-Δ1 ura3-52 gal2 gcn4-Δ1*
KY803-1	Isogenic to KY803 except *ccr4::URA3*
KY803-c1	Isogenic to KY803 except *caf1::LEU2*
MY8	Isogenic to KY803 except *not1-2*
MY8-1d	Isogenic to MY8 except *ccr4::URA3*
MY8-c1c	Isogenic to MY8 except *caf1::LEU2*
MY1737	Isogenic to KY803 except *his3::TRP1 not1::LEU2* pRS426-NOT1(396–2108)
MY1738	Isogenic to KY803 except *MATα not1::LEU2* pRS426-NOT1(1319–2108)
MY16	Isogenic to KY803 except *not2-1*
MY16-1c	Isogenic to MY16 except *ccr4::URA3*
MY508	Isogenic to KY803 except *not3::URA3*
MY508-c1b	Isogenic to MY508 except *caf1::LEU2*
MY25	Isogenic to KY803 except *not3-2*
MY25-1b	Isogenic to MY25 except *ccr4::URA3*
MY537	Isogenic to KY803 except *not4::URA3*
MY537-1	Isogenic to MY537 *not4::ura3*
MY537-1-1b	Isogenic to MY537-1 *ccr4::URA3*
MY1735	Isogenic to KY803 except *not5::URA3*
EGY188	*MATa ura3 his3 trp1 LexA-LEU2*
EGY188-1	Isogenic to EGY188 except *ccr4::URA3*
EGY188-c1	Isogenic to EGY188 except *caf1::URA3*
EGY188-1-1	Isogenic to EGY188 except *ccr4::ura3*
EGY188-c1-1	Isogenic to EGY188 except *caf1::ura3*
787-6b	*MATα adh1-11 ADR1-5^{C}-TRP1 ura3 his3 leu2 trp1*
1076-2c-1	*MATα ura3 his3 leu2 trp1 ccr4::ura3::TRP1*
1278-5d	*MATa ura3 his3 leu2 trp1 caf1::LEU2*
1393-4a	*MATα his3/his2 leu2 ura3 not2::TRP1*
1402-1a	*MATα ura3 his3 leu2 trp1 not4::URA3*
1402-4a	*MATα ura3 his3 leu2 trp1 not4::URA3*
1422-21	*MATα adh1-11 ura3 his3 leu2 trp1 not1::LEU2* pRS426-NOT1(1490–2108)
1469-2-1c	*MATa ura3 his3 leu2 trp1 ccr4::ura3::TRP1*
1462-3c	*MATa ura3 his3 leu2 trp1 not5::URA3*

NOT1 (residues 667 to 1152) binds CCR4 and CAF1, whereas the C-terminus of NOT1 (1490 to 2108) associates with NOT2, -4, and -5. We provide evidence that the arrangement of the proteins in the complex is CCR4-CAF1-NOT1-(NOT2, NOT5), with NOT3 and NOT4 peripheral to NOT2 and NOT5. Further, the physical separation of CCR4 and CAF1 from the other NOT proteins correlates, in general, with phenotypes associated with defects in CCR4 and CAF1 compared to the other *NOT* mutations. The CCR4-NOT complex appears, therefore, to be composed of at least two physically separate groups of proteins that can function differently depending on the promoter context.

MATERIALS AND METHODS

Yeast strains, growth conditions, and enzyme assays. Yeast strains (Table 1) were grown at 30°C on YEP medium (2% yeast extract, 1% Bacto Peptone) or selective medium (7) supplemented with 5% glucose or with 2% galactose and 2% raffinose unless otherwise indicated. β-Galactosidase assays and alcohol dehydrogenase (ADH) assays were carried out as described previously (12). Assay values represent the averages of at least three independent assays. The yeast transformation protocol was as described previously (7, 17).

DNA constructions. The LexA-NOT1 plasmids containing various length of *NOT1* were constructed as follows. For expression of LexA-NOT1(667–1152), pLexA-NOT1 was cut with *Bam*HI and *Xho*I and the segment carrying codons 667 to 1152 was ligated with pLexA202-4 (7) cut with the same two enzymes. For expression of LexA-NOT1(1480–2108), pET28a-NOT1 (*Xba*I-*Sal*I) was cut with *Bam*HI and *Sal*I and the segment carrying codons 1480 to 2108 was inserted into the *Bam*HI and *Sal*I sites of pLexA-202-2. For expression of LexA-NOT1(1–667), pLexA-NOT1 was cut with *Bam*HI and *Sal*I, the ends were filled in with the large subunit of *Escherichia coli* DNA polymerase (Klenow), and the plasmid was religated. For expression of LexA-NOT1(1–1152), pLexA-NOT1 was cut with *Eco*RI and *Xho*I and the fragment encoding residues 1 to 1152 of NOT1 was ligated with pLexA202-2 cut with *Eco*RI and *Sal*I. For expression of LexA-NOT1(667–2108), pLexA-NOT1 was cut with *Bam*HI and *Sal*I and the piece encoding residues 667 to 2108 was inserted into the *Bam*HI and *Sal*I sites of pLexA202-2. Expression of all fusion proteins was confirmed by Western blot analysis.

Antibodies and immunoprecipitation. For Western analysis, the antibodies were directed against glutathione *S*-transferase (GST)–CAF1, GST-NOT2, His_6-NOT5, GST-NOT1(1480–2108), and GST-DHH1(267–506) fusion proteins. Western analysis was conducted as described previously (13). Immunoprecipitations were carried out as previously described (12). The CAF1 antibody was partially purified as described elsewhere (14).

Gel filtration chromatography. The procedure for gel filtration chromatography using a Superose 6 10/30 column was performed as described in detail elsewhere (20) except that the running buffer consisted of 50 mM Tris, 150 mM potassium acetate, and 0.02% Tween 20 only. The flow rate was 0.2 ml/min, and 0.5 ml was collected per fraction. Molecular weights for each fraction were calculated based on the elution volumes of blue dextran (7.5 ml), thyroglobin (12 ml), and bovine serum albumin (16 ml).

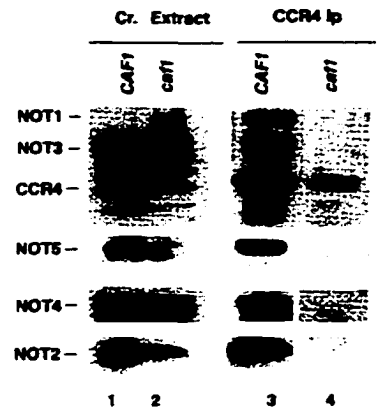
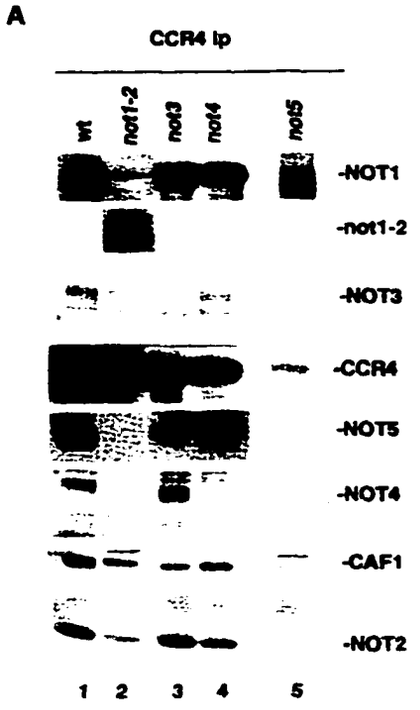


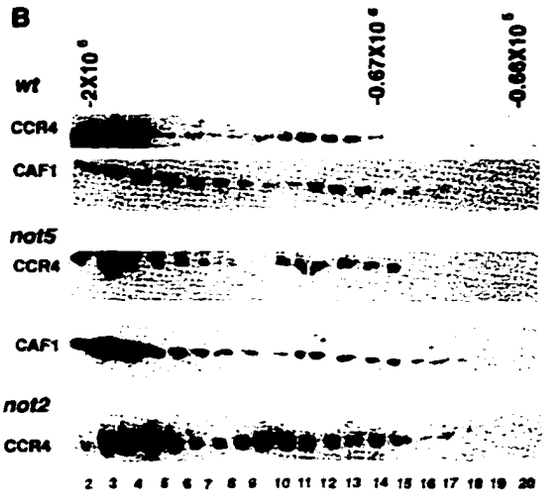
FIG. 1. CCR4 requires CAF1 to immunoprecipitate NOT proteins. Immunoprecipitations with CCR4 antibody were conducted in *caf1* (A792, *pop2*) and wild-type (A790) strains. Lanes 1 and 2, protein extracts from strains A790 and A792, respectively; lanes 3 and 4, immunoprecipitated (Ip) proteins analyzed by Western analysis using antibodies directed against NOT1 through NOT5 and CCR4. The NOT1 antibody used in these experiments could not detect NOT1 protein in crude (cr.) extracts (lanes 1 and 2), but other results indicate that NOT1 is present in both *CAF1*- and *caf1*-containing strains (20).

RESULTS

CAF1 is required for CCR4 association with the NOT proteins. We had shown previously that deleting *CAF1* removed CCR4 completely from the 1-mDa CCR4-NOT complex and reduced significantly but did not eliminate CCR4 association in the 1.9-mDa complex (20). We have also shown that CCR4 is dependent on CAF1 in its association with NOT1 and NOT2 (20). Using a complete deletion of *CAF1*, we further investigated the dependency on CAF1 for CCR4 for interaction with the other NOT proteins. In a wild-type strain, immunoprecipitation of CCR4 with anti-CCR4 antibody brought down NOT1 through NOT5 (Fig. 1, lane 3). In contrast, in a *caf1* deletion strain, none of the NOT proteins coimmunoprecipitated with CCR4 (lane 4), although all NOT proteins were present in the crude extract (lane 2). CAF1 protein is therefore required for CCR4 to associate with all NOT proteins.

Physical interactions of CCR4-CAF1-NOT1 are independent of the NOT3, NOT4, and NOT5 proteins. Since the above results suggest that the arrangement of proteins is CCR4-





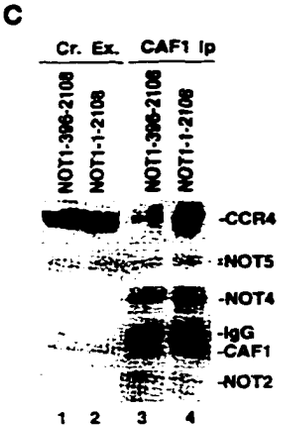


FIG. 2. CCR4 associates with CAF1 in the absence of *NOT3*, *NOT4*, *NOT5*, and the N-terminal 396 residues of NOT1. (A) Immunoprecipitations (Ip) were conducted with anti-CCR4 antibody. Western analysis was conducted with antibody directed against NOT1, NOT3, CCR4, NOT4, CAF1, NOT2, or NOT5 as indicated. An enhanced chemiluminescence-based system was used for NOT1, CCR4, and NOT5 Western blots for lanes 1 to 4, whereas an alkaline phosphatase-based system was used for the remainder of the results. Strains: wild type (wt), KY803; *not1-2*, MY8; *not3*, MY508; *not4*, MY537; *not5*, MY1735. (B) Yeast extracts from KY803 (wild type [wt]), 1393-4a (*not2*), and MY1735 (*not5*) were analyzed by gel filtration chromatography using a Superose 6 10/30 column. The protein extracts were precleared by centrifugation at 100,000 × *g* for 1 min, and 200 μl of sample was loaded onto the column. The flow rate was 0.2 ml/min, and a 0.5-ml volume was collected in each fraction; 100 μl from each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using CCR4 and CAF1 antibodies. Molecular weight markers for the gel filtration experiment were blue dextran (2×10^6 Da), thyroglobulin (0.67×10^6 Da), and bovine serum albumin (6.6×10^4 Da). (C) Immunoprecipitations were conducted in strain MY1737 [*not1* pNOT1(396–2108)] and wild-type backgrounds with CAF1 antibody. Lanes 1 and 2 contain 1/10 of the crude extract (Cr. Ex.) protein input used for the immunoprecipitations (Ip) in lanes 3 and 4. Western analysis was conducted with anti-CCR4 and anti-NOT antibodies as indicated. NOT4 and NOT2 proteins in the crude extracts in lanes 1 and 2 were visible in the original Western blots and were in equal abundance for the two strains. IgG, immunoglobulin G.

CAF1-NOTs, we subsequently examined what factors were required for CCR4, CAF1, and NOT1 to associate. Immunoprecipitation of CCR4 showed that NOT1 and CAF1 can be coimmunoprecipitated in *not3*-, *not4*-, or *not5*-deleted backgrounds (Fig. 2A, lanes 3 to 5). (For lane 5, the CAF1 protein was clearly visible in the original Western results.) The same results were obtained when anti-CAF1 antibody was used for immunoprecipitation (data not shown). The observation that the CCR4-CAF1 interaction was not dependent on NOT5 was further confirmed by gel filtration analysis (Fig. 2B, middle panel). In a *not5* strain, CCR4 and CAF1 cofractionated in both 1.9-mDa (fractions 3 and 4) and 0.8-mDa (fractions 11 to 13) complexes which have been previously described for CCR4 and CAF1 (20) (Fig. 2B, top panel, in which CCR4 migrates in fractions 3 and 4 and fractions 10 to 12). It should be noted that the smaller complex in the *not5* strain runs at a slightly smaller size (0.8 mDa) than the wild-type strain (0.9 mDa), probably due to loss of the NOT5 and other potential proteins. Also, the 0.9-mDa CCR4-NOT complex observed for the wild-type strain runs at slightly smaller size than in the strains used in our previous study (20).

We also conducted gel filtration analysis in *not3* and *not4* backgrounds. In a *not3* deletion strain, CCR4 and CAF1 still migrated in large and medium complexes but the proteins were clearly more spread out, suggesting that their stability in the complexes was being compromised (data not shown). A *not4* deletion had no apparent effect on CCR4 or CAF1 migration in the 1.9- and 0.8-mDa complexes (data not shown). CCR4

and CAF1 can therefore still associate in the CCR4-NOT complex in the absence of *not3*, *not4*, or *not5*, although these deletions may cause subtle effects on the structure and integrity of the complex. It should also be mentioned that in contrast to CCR4 and CAF1, the association of several other components of the CCR4-NOT complex could not be determined by gel filtration chromatography. NOT2 protein was hardly detectable after Superose 6 chromatography, NOT3 tended to migrate at its monomeric size, and NOT5 and NOT1 did not tend to migrate in well-defined peaks as was observed for CCR4 and CAF1 (data not shown).

The above results indicate that CCR4 and CAF1 can associate with NOT1 in the absence of NOT3, NOT4, or NOT5. In a *not2* strain, however, the association of CCR4 with CAF1 and the NOT proteins could not be ascertained by immunoprecipitation due to the inability to immunoprecipitate sufficient levels of CCR4 and CAF1 proteins. This may be partially the result of the very low amount of CAF1 and several of the NOT proteins present in the extracts (data not shown; see also Fig. 5), but it may also result from overall instability of the complex and susceptibility to proteolytic degradation in a *not2* background. Our gel filtration analysis indicated that CCR4 still migrated in 1.9- and 0.9-mD complexes in a *not2* background (bottom panel of Fig. 2B), but the presence of CAF1 in the 0.9-mDa complex could not be determined and only a very small amount of CAF1 was visible in the 1.9-mDa complex (data not shown). In a *not2-1* strain background, CCR4 and CAF1 migrated in both 1.9- and 0.9-mDa complexes (data not shown). These results suggest that NOT2 affects the overall integrity of the complex but may not be required for CCR4 association in the CCR4-NOT complex.

It is also apparent in Fig. 2A that when CCR4 is immunoprecipitated, NOT4 is not required for NOT2, -3, and -5 to associate with CCR4, CAF1, and NOT1, and similarly, NOT3 is not required for NOT2, -4, -5, to associate with CCR4, CAF1, and NOT1. In a *not5* background, however, because of the decrease in abundance of NOT3, NOT4, and NOT2 protein levels in the crude extracts used for the immunoprecipitation (data not shown), we could not ascertain if these three NOT proteins associated with CCR4, CAF1, and NOT1 following the immunoprecipitation. These results confirm that CCR4 and CAF1 are tightly if not directly linked, that NOT3 to NOT5 are not required for CCR4 and CAF1 association or with their association with NOT1, and that neither NOT3 nor NOT4 is required for NOT2 and NOT5 association with CCR4, CAF1, and NOT1.

The C terminus of NOT1 is not required for CCR4 and CAF1 association. We used several complementary approaches to identify the region of NOT1 which interacted with CCR4 and CAF1. Since a *not1* deletion is lethal, we initially used two truncated versions of NOT1 to assess the NOT1 requirement for CCR4 and CAF1 association. First, we analyzed the *not1-2* allele. The *not1-2* allele results in a NOT1 protein that is about 120 kDa in size (Fig. 2A, lane 2) and has been reported to be the result of a stop codon located in the region between residues 396 and 1318 of NOT1 (26). About 10% of the not1-2 protein is full length, which is apparently sufficient for the yeast to survive. The truncated not1-2 protein still coimmunoprecipitated with either CCR4 or CAF1 (Fig. 2A, lane 2, and data not shown). However, NOT5 and NOT4 no longer immunoprecipitated with CCR4 in a *not1-2* strain (Fig. 2A, lane 2), and the amount of NOT2 was significantly reduced in the immunoprecipitation. The reduced amount of NOT2 that coimmunoprecipitated could be derived from NOT2 binding to the full-length NOT1 protein (Fig. 2A, lane 2). NOT5, NOT2, and NOT4 appear, therefore, to interact with the C-terminal region of NOT1, a result confirmed by other results described below.

Second, we examined whether the CCR4-CAF1 interaction required the N-terminal 395 codons of NOT1. Immunoprecipitating CAF1 coimmunoprecipitated a significant amount of CCR4 in a strain carrying NOT1(396–2108) (Fig. 2C, lane 3). The central region of NOT1 (residues 396 to about 1100) appears sufficient, therefore, for CCR4 interaction with CAF1, although the N-terminal 395 residues of NOT1 appear to aid the stable association of CCR4 with CAF1 (Fig. 2C; compare lane 3 with lane 4).

An internal segment (residues 667 to 1152) of NOT1 is sufficient for binding CCR4 and CAF1. To examine more thoroughly the region of NOT1 that interacted with CCR4 and CAF1, we expressed in yeast several LexA-NOT1 fusions and determined their ability to be coimmunoprecipitated with CCR4 and CAF1. Three LexA-NOT1 fusions, LexA-NOT1(667–1152), LexA-NOT1(667–2108), and LexA-NOT1(1–1152), were coimmunoprecipitated with CCR4 (Fig. 3A, lanes 6, 9, and 10) or CAF1 antibody (data not shown). In contrast, the LexA-NOT1(1–667) and LexA-NOT1(1490–2108) could not be coimmunoprecipitated with CCR4 or CAF1 (Fig. 3A, lanes 7 and 8, and data not shown). Residues 667 to 1152 of NOT1 are, therefore, sufficient for binding CCR4 and CAF1, a conclusion that agrees with the ability of CCR4 and/or CAF1 to immunoprecipitate both the truncated not1-2 protein and NOT1(396–2108).

The N-terminal 1318 residues of NOT1 are required for association of CCR4 with CAF1 in the 0.9-mDa complex. We further analyzed CCR4-CAF1-NOT1 interactions by Superose 6 gel filtration. Removing the N-terminal half of NOT1 (residues 1 to 1318) caused the dissociation of CCR4 from the 0.9-mDa complex peak fractions 10 to 12 (compare Fig. 4B with Fig. 4A), suggesting that residues 1 to 1318 of NOT1 are required for the physical integrity of CCR4 in this complex. Very little CAF1 protein could be detected following Superose 6 chromatography (data not shown). The total CAF1 protein level was reduced in the strain carrying NOT1(1318–2108) (Fig. 5), but that reduction alone cannot explain the extremely low level of total CAF1 in all Superose 6 fractions. The CAF1 protein may be particularly sensitive to degradation without the presence of the N terminus of NOT1. Deleting the N-terminal 395 codons of NOT1 had no effect on CCR4 migration in either the 1.9- or 0.9-mDa complex (Fig. 4C). The gel filtration profile of CCR4 in the *not1-2* strain showed that CCR4 migrated at 1.9 mDa and about 700 kDa (fractions 12 to 14). The shift of the 0.9-mDa peak may be the result of both the truncated not1-2 protein being about 70 kDa smaller than NOT1 and the loss of the NOT5 and NOT4 proteins (Fig. 2A). CAF1 was also found to migrate in 1.9- and 0.9-mDa peaks in the *not1-2* strain and in 1.9- and 0.9-mDa complexes in the *NOT1(396–2108)* strain (data not shown). These gel filtration results agree with the above immunoprecipitation results and indicate that in order for the CCR4-CAF1-NOT1 proteins to associate in the 0.9-mDa complex, NOT1 must contain residues 396 to about 1100.

The C-terminal region from residues 1490 to 2108 of NOT1 interacts with NOT2, NOT4, and NOT5. Because the C-terminal part of NOT1 was required for NOT5, NOT4, and NOT2 to associate with CCR4, CAF1, and NOT1 (Fig. 2A, lane 2), we initially used two-hybrid analysis to examine interactions of LexA-NOT1(1490–2108) with the NOT components of the CCR4-NOT complex (20). B42-NOT2, B42-NOT4, and B42-NOT5 all interacted with LexA-NOT1(1490–2108) (410, 520, and 77 U of β-galactosidase/mg, respectively). As expected from the immunoprecipitation analysis, neither B42-CAF1 nor

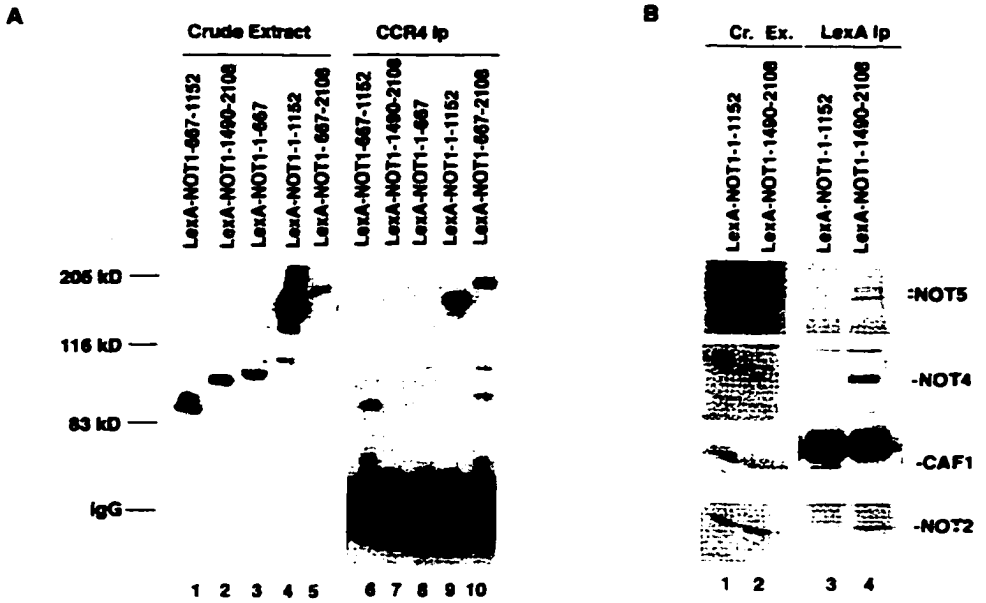


FIG. 3. Localization of the NOT1 protein region that is sufficient for binding CCR4. (A) LexA-NOT1(667–1152) is sufficient for binding to CCR4 and CAF1. LexA-NOT1 fusions as indicated were expressed in strain EGY188, and immunoprecipitations were conducted with anti-CCR4 antibody. Western analysis was conducted with anti-LexA antibody. The crude protein extracts in lanes 1 to 5 contain 1/10 of the amount of extract used for the immunoprecipitations (Ip) displayed in lanes 6 to 10, respectively. IgG, immunoglobulin G. (B) The C terminus of NOT1 binds NOT2, NOT4, and NOT5. Strain EGY188 containing either LexA-NOT1(1–1152) or LexA-NOT1(1490–2108) was immunoprecipitated with LexA antibody. Western analysis using the antibodies as indicated was conducted as detailed in Fig. 1. Cr. Ex., crude extract.

B42-CCR4 interacted with the C-terminal region of NOT1 (5.5 and 3.0 U of β-galactosidase/mg, respectively; values for B42-NOT1, B42-NOT3, and B42 were 5.8, 4.5, and 3.2 U of β-galactosidase/mg, respectively). To further analyze the interaction of the C terminus of NOT1 with other components of the CCR4-NOT complex, we immunoprecipitated LexA-NOT1(1–1152) and LexA-NOT1(1490–2108) with LexA antibody. As displayed in Fig. 3B, lane 4, the C-terminal portion of NOT1(1490–2108) was able to immunoprecipitate NOT2, NOT5, and NOT4. In contrast, LexA-NOT1(1–1152) did not immunoprecipitate these proteins and instead immunoprecipitated CAF1 (Fig. 3B, lane 3) and a small amount of NOT3 (not shown). While CCR4 did not immunoprecipitate with either LexA fusion, in Fig. 3A it clearly interacted with residues 667 to 1152 to NOT1. The above immunoprecipitation, gel filtration, and two-hybrid analysis indicate, therefore, that the NOT1 protein contains two separable domains, 667 to 1152 for binding CAF1 and CCR4 and 1490 to 2108 for interacting with NOT2, NOT4, and NOT5.

The effects of deleting the different regions of *NOT1* on *ADH2* expression were subsequently analyzed. NOT1(1318–2108) resulted in a two- to threefold decrease in ADH II activity compared to NOT1(396–2108) or full-length NOT1 (Table 2). LexA-NOT1(1490–2108) resulted in a similar low level of *ADH2* expression (Table 2). Coexpressing in yeast LexA-NOT1(1–1152) along with NOT1(1318–2108) allowed a twofold increase in ADH II activity. In contrast, coexpressing LexA-NOT1(1–667) was insufficient for recovering the ability of NOT1(1318–2108) to fully activate *ADH2*. The 667–1152 region of NOT1 that binds CAF1 and CCR4 appears, there-

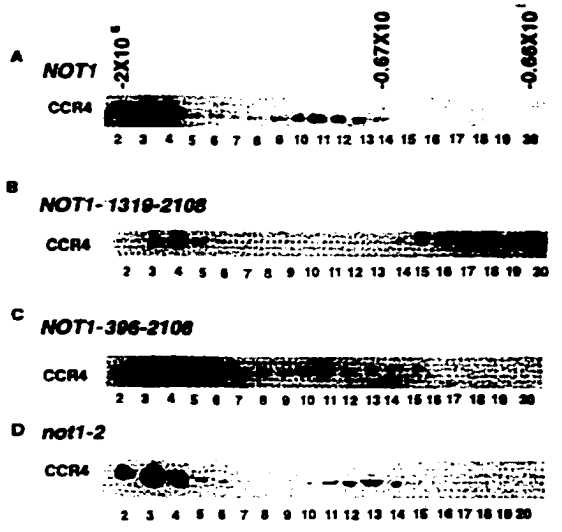


FIG. 4. Gel filtration analysis of CCR4 in *NOT1* mutant backgrounds. (A) Strain KY803 (wild type); (B) strain MY1738 [*not1* pNOT1(1319–2108)]; (C) strain MY1737 [*not1* pNOT1(396–2108)]; (D) strain MY8 (*not1-2*). Gel filtration chromatography was conducted as described for Fig. 2B. Anti-CCR4 antibody was used to detect the CCR4 protein.

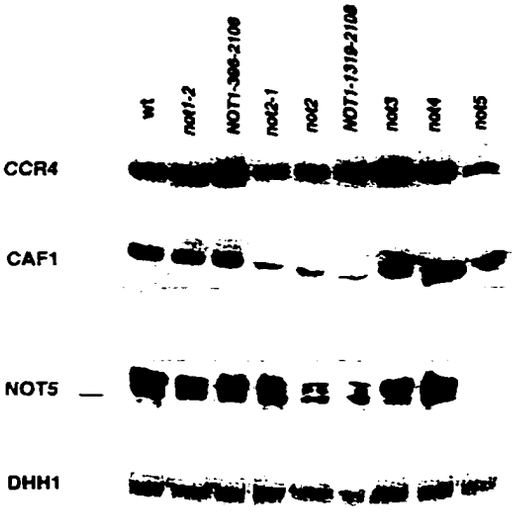


FIG. 5. CCR4, CAF1, and NOT5 protein levels in *not* mutant backgrounds. All strains were grown to mid-log phase in YEP medium containing 5% glucose. Cells were harvested and lysed, and 40 μg of total protein was loaded in each lane. The RNA helicase homolog DHH1 was used as an internal control to demonstrate equivalent loading on the sodium dodecyl sulfate-polyacrylamide gel. Strains used: KY803 (wild type [wt]); MY8 (*not1-2*); MY1737 [*not1* pNOT1 (396–2108)]; MY1738 [*not1* pNOT1(1319–2108)]; MY16 (*not2-1*); 1393-4a (*not2*); MY508 (*not3*); MY537 (*not4*); MY1735 (*not5*). Western blot analysis was conducted with antibodies directed against CCR4, CAF1, NOT5, and DHH1 as indicated.

fore, to be necessary for recovering in *trans* NOT1(1318–2108) function and confirms the existence of two distinct functional regions of NOT1.

NOT2 and NOT5 associate closely and in the absence of CAF1, NOT3, or NOT4. NOT5-NOT2 interactions were also analyzed following the observation that when LexA-NOT5 is immunoprecipitated with anti-LexA antibody, all components of the CCR4-NOT complex can be coimmunoprecipitated (Fig. 6A, lane 1). Immunoprecipitation of LexA alone does not coimmunoprecipitate any of these proteins (reference 20 and data not shown), indicating that it is the NOT5 moiety which is interacting with these proteins. As shown in Fig. 6A, lanes 2 to 4, NOT2 was capable of immunoprecipitating with LexA-NOT5 in the absence of CAF1, NOT3, or NOT4. As expected no CCR4 was capable of coimmunoprecipitating with LexA-NOT5 in the absence of CAF1 (Fig. 6A, lane 2). It should be noted that in the original results (Fig. 6A, lane 3) some CCR4 protein was immunoprecipitated with LexA-NOT5 in a *not3* strain. These results are in agreement with the physical separation of CAF1 and CCR4 from the NOT2 and NOT5 proteins and the dependency on CAF1 for CCR4 association with these other factors. Moreover, NOT3 and NOT4 had no effect on the ability of LexA-NOT5 to coimmunoprecipitate CAF1, NOT2, or NOT1, although NOT3 may play a role in stabilizing CCR4 interactions with the complex. We subsequently used anti-NOT5 antibody to analyze more completely the NOT5-NOT2 association. Anti-NOT5 antibody immunoprecipitated only NOT5, NOT2, and a small amount of NOT1 (Fig. 6B, lane 2). While the NOT5 antibody may interfere with the association of NOT5 with the rest of the CCR4-NOT protein components, its ability to immunoprecipitate NOT2 confirms a close physical association of NOT2 and NOT5.

NOT3 associates with the CCR4-NOT complex independently of CCR4, CAF1, or NOT4. Immunoprecipitating NOT3 was also found to coimmunoprecipitate the whole CCR4-NOT complex (Fig. 6C, lane 1). We therefore investigated the effects of various deletions in CCR4-NOT components on NOT3 immunoprecipitation of the complex. Deleting *CCR4* had no effect on NOT3 associations (Fig. 6C, lane 2), whereas *caf1*, as expected, resulted in only CCR4 not being able to associate in the complex (lane 3). Deleting NOT4 also had no effect on NOT3 interaction with the other components of the complex (Fig. 6C, lane 4). In *not1-2*, *not2*, or *not5* strains, NOT3 could not be immunoprecipitated, suggesting that NOT2, NOT5, and the C-terminal region of NOT1 are required for stable NOT3 association with the rest of the complex or existence in an immunoprecipitable form.

CAF1 and CCR4 can act phenotypically opposite NOT2, NOT4, and NOT5. Previously we had shown that a *caf1* or *ccr4* deletion resulted in no or very little increased 3-AT resistance (20) whereas mutation or deletion of the *NOT* genes is known to cause increased 3-AT resistance, indicative of increased *HIS3* gene expression (6, 23). The *not4* and *not5* deletions also resulted in increased *ADR1-5^C* activation of *ADH2* under glucose growth conditions (ADH II activities of 370 ± 24 and 220 ± 10 mU/mg, respectively, versus 94 ± 7.6 mU/mg for *ADR1-5^C*), whereas a *caf1* deletion had no effect on *ADR1-5^C* activation of *ADH2* and a *ccr4* deletion reduced twofold the ability of *ADR1-5^C* to activate (ADH II activities of 95 ± 7.4 and 45 ± 1.4 mU/mg, respectively). Similar differences between the effects of *caf1* and *ccr4* effects on gene expression and the effects of the *not* alleles were reported previously (20). The most salient of these is the reduction in *HO-lacZ* and *FKS1-lacZ* expression caused by *ccr4* and *caf1* alleles and the two- to threefold increases in *HO-lacZ* or *FKS1-lacZ* expression caused by *not1-2*, *not2*, and *not4* defects (20). These phenotypic effects support the existence of the separate location of these groups of proteins within the CCR4-NOT complex.

Since deleting *NOT1* is lethal, we examined the effects of deleting components from the two separate groups of proteins in the CCR4-NOT complex. Suitable crosses were made between either *ccr4* or *caf1* deletions and *not* deletions, and the viability of different deletion combinations was analyzed by tetrad analysis. As shown in Table 3, *not2* and *not5* were lethal in combination with either *ccr4* or *caf1*. Lethality was con-

TABLE 2. Effects of *not1* truncations on *ADH2* expression[a]

NOT1 plasmid	LexA plasmid	ADH II activity (mU/mg; mean ± SEM)[b]
NOT1(396–2108)	None	2,900 ± 450
NOT1(1319–2108)	None	910 ± 150
	LexA-NOT1(1–2108)	2,300 ± 150
	LexA-NOT1(1–1152)	1,800 ± 120
	LexA-NOT1(1–667)	830 ± 100
None	LexA-NOT1(1–2108)	2,200 ± 320
	LexA-NOT1(1490–2108)	1,000 ± 230

[a] ADH II assays were conducted after growth of strain 1422-21 on YEP medium containing 3% ethanol. ADH II activities in a strain carrying an integrated full-length *NOT1* gene is generally 2,500 to 3,000 mU/mg. LexA plasmids contain *NOT1* sequences fused to *LexA-202* (7). NOT1 plasmids were pRS426 derivatives.

[b] Average of at least three separate transformants.

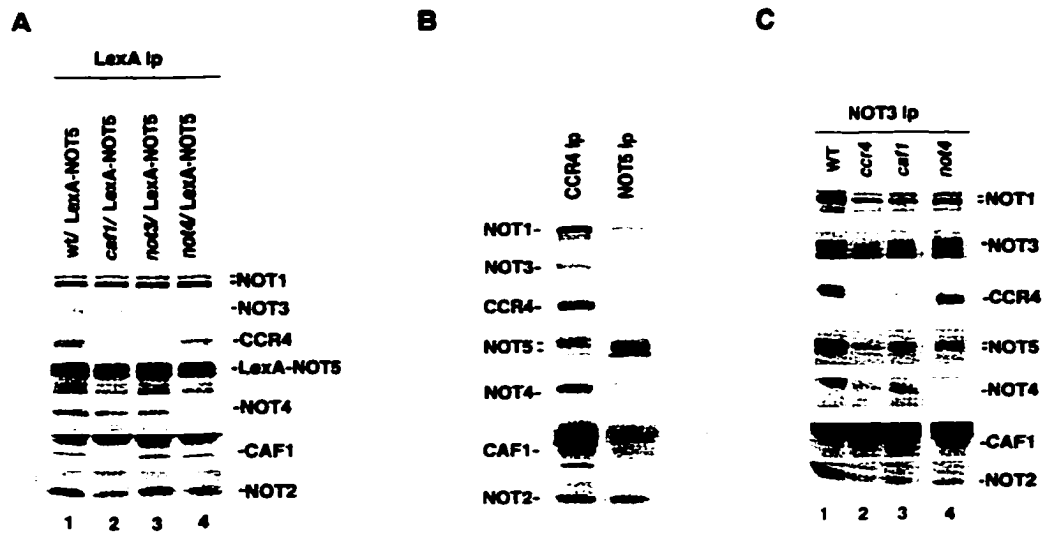


FIG. 6. NOT5 coimmunoprecipitates with NOT2 in the absence of CAF1, NOT3, and NOT4. (A) LexA-NOT5 immunoprecipitates NOT2 in the absence of CAF1, NOT3, and NOT4. Immunoprecipitations (Ip) were conducted with anti-LexA antibody, and Western analysis used antibodies as indicated; LexA-NOT5 is full-length NOT5 fused to LexA(1–202). Lanes: 1, KY803 (wild type [wt]); 2, KY803-c1 (*caf1*); 3, MY508 (*not3*); 4, MY537 (*not4*). In the original Western blots, CCR4 was immunoprecipitated in lane 3. (B) NOT5 antibody immunoprecipitates NOT2. Antibody against CCR4 (lane 1) or NOT5 (lane 2) was used for immunoprecipitations from strain KY803. (C) NOT3 immunoprecipitates NOT1, NOT2, and NOT5 in the absence of CCR4, CAF1, and NOT4. Anti-NOT3 antibody was used to conduct the immunoprecipitations. WT (wild type), strain KY803; *ccr4*, KY803-1; *caf1*, KY803-c1; *not4*, MY537. Western analysis was conducted as detailed above.

firmed in all cases by ascertaining whether a plasmid-borne copy of one of the deleted genes could rescue the lethality. In addition, in all cases where the lethality was rescued by the plasmid-borne gene, the plasmid could not be lost from the cell, confirming the lethality of the double deletion. These synthetic lethalities are consistent with the importance of NOT2 and NOT5 to the integrity and function of the CCR4-NOT complex and to a role that is in addition to and/or separate from CCR4 and CAF1. Whereas deleting *not3* did not result in synthetic phenotypes with either *caf1* or *ccr4*, the *not3-2* mutation clearly resulted in exacerbated growth phenotypes with *ccr4* (Table 3). *not1-2* also displayed synthetic growth defects with a *ccr4* or *caf1* deletion (Table 3), consistent with the observation that in a *not1-2* strain, NOT2, NOT4, and NOT5 associate in the complex less well due to the increased levels of C-terminally truncated NOT1 protein (Fig. 2A, lane 2).

The hallmark of the *not* alleles is their increased resistance to 3-AT, yet the *ccr4* and *caf1* alleles do not display this phenotype (20). Because of the possible antagonistic behavior of CCR4 and CAF1 in relation to the other NOT proteins, we tested the effect of a *ccr4* or *caf1* deletion on the ability of *not* alleles to confer increased 3-AT resistance. We observed that a *caf1* or *ccr4* deletion suppressed the increased 3-AT resistance of *not1-2*, *not3-2*, and *not4* alleles (Table 4), confirming that CCR4 and CAF1 can act in an opposite manner to the NOT proteins in certain promoter contexts.

DISCUSSION

CCR4-CAF1 interact with the N-terminal 1152 residues of NOT1, whereas NOT2, NOT4, and NOT5 interact with the C-terminal 1490–2108 region of NOT1. It was shown previously that disrupting the *CAF1* gene blocked the ability of CCR4 to associate with NOT1 (20). In this report, we showed that CAF1 is required for CCR4 to associate with all of the NOT proteins. *NOT3*, *NOT4*, and *NOT5* were, in turn, found not to be required for CCR4-CAF1-NOT1 association or for CCR4 and CAF1 association in the 1-mDa complex. Relatedly, immunoprecipitating NOT3 or LexA-NOT5 did not coimmunoprecipitate CCR4 when *CAF1* was deleted. These results clearly indicate that CCR4 binds through CAF1 to associate with NOT1 and the other components of the complex (Fig. 7 summarizes the interactions in the CCR4-NOT complex).

The region of NOT1 with which CCR4 and CAF1 physically associate was found to be distinct from that bound by the NOT2, NOT4, and NOT5 proteins. Several lines of evidence indicate that the region of NOT1 binding CAF1 is localized to residues 667 to 1152. First, CCR4 or CAF1 could immunoprecipitate an internal segment of NOT1 (residues 667 to 1152). Second, when the immunoprecipitation was conducted in the reverse direction, LexA-NOT1(1–1152) immunoprecipitated CAF1 but LexA-NOT1(1490–2108) did not. Third, in a *not1-2* strain wherein the major NOT1 species contains only the N-terminal 1,000 residues or so of NOT1, both CCR4 and CAF1 immunoprecipitated with the not1-2 protein. Fourth, removing the N-terminal 395 residues of NOT1 did not abrogate the ability of CAF1 to immunoprecipitate CCR4, although the N-terminal segment of NOT1 clearly played some role in stabilizing the CCR4-CAF1 interactions. Finally, when the N-terminal 1,318 residues of NOT1 were removed, neither CCR4 nor CAF1 was able to associate in the 1-mDa complex.

In contrast to the above results, the region of NOT1 that associates with NOT2, NOT4, and NOT5 was localized to the C-terminal portion of NOT1 (residues 1490 to 2108). The NOT2, NOT4, and NOT5 were found to display two-hybrid interactions with LexA-NOT1(1490–2108), and immunopre-

TABLE 3. Synthetic lethalities between deletions in *ccr4* and *caf1* and mutations in the *not* genes[a]

Construct	Growth		
	wt	*ccr4*	*caf1*
wt	+	+	+
not1-2			
30°C	+	+	+
34°C	+	−	−
pNOT1(1490–2108)			
not1	+	Lethal	ND
not2	+	Lethal	Lethal
not2-1			
30°C	+	+	ND
34°C	+	−	ND
not3	+	+	+
not3-2			
30°C	+	−	ND
37°C	+	−	ND
not4	+	+	+
not5	+	Lethal	Lethal

[a] Growth was determined at 30°C on YEP medium supplemented with 2% glucose unless otherwise indicated. +, growth; −, no growth; lethal, the gene pair resulted in cell death; ND, not done. Isogenic strains used: wild type (wt), KY803; *not1-2*, MY8; *not1-2 ccr4*, MY8-1d; *not1-2 caf1*, MY8-c1c; *not3*, MY508; *not3 caf1*, MY508-c1b; *not2-1*, MY16; *not2-1 ccr4*, MY16-1c; *not3*, MY508; *not3 caf1*, MY508-c1b; *not3-2*, MY25; *not3-2 ccr4*, MY25-1b; *not4*, MY537-1; *not4 ccr4*, MY537-1-1b. *not3 ccr4* segregants were obtained from 1076-2c-1 × MY508 crosses, and *not4 caf1* segregants were obtained from 1278-5d × 1402-1a and 1278-5d × 1402-4a crosses. pNOT1(1480–2108) *not1 ccr4* lethality was confirmed by analysis of segregants of diploid 1471 [1422-21/pNOT1(1480–2108)/pLexA-NOT1 × 1469-2-1c], *ccr4 not2* lethality was confirmed from strain 1441 (188-1/pLexA-NOT2 × 1393-4a), *ccr4 not5* lethality was confirmed from strain 1469 (1076-2c-1/pLexANOT5 × 1462-3a), *caf1 not2* lethality was confirmed from strain 1442 (188-c1/pLexA-CAF1 × 1393-4a), and *caf1 not5* lethality was confirmed from strain 1469 (1076-2c-1/pLexA-NOT5 × 1462-3c).

cipitating LexA-NOT1(1490–2108) coimmunoprecipitated NOT2, NOT4, and NOT5. As a comparison, immunoprecipitating LexA-NOT1(1–1152) failed to coimmunoprecipitate these proteins. Moreover, in the *not1-2* strain, NOT5 and NOT4 did not immunoprecipitate with CCR4 or CAF1 and only a limited amount of NOT2 coimmunoprecipitated (possibly due to association with the residual full-length NOT1 still present in the cell). Finally, NOT2, NOT4, and NOT5 were coimmunoprecipitated in the absence of CAF1 when either

TABLE 4. *caf1* and *ccr4* disruptions suppress *not*-induced 3-AT phenotypes[a]

Relevant genotype	Growth on 3-AT (mM)		
	0	5	10
Wild type	+	−	−
caf1	+	−	−
ccr4	+	−	−
not1-2	+	+	+
not1-2 caf1	+	w	−
not1-2 ccr4	+	−	−
not3-2	+	+	+
not3-2 ccr4	+	−	−
not4	+	−	+
not4 caf1	+	−	−
not4 ccr4	+	−	−

[a] All strains are isogenic to KY803 except for the indicated allele. Growth was monitored on minimal medium lacking histidine and supplemented with 3-AT as indicated.

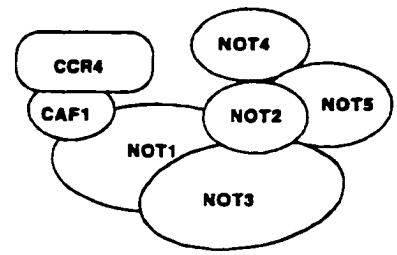


FIG. 7. Model for protein contacts in the CCR4-NOT complex. Based on the results presented herein, CAF1 is presumed to bind to residues 667 to 1152 of NOT1, CCR4 binds to CAF1, and NOT2 and NOT5 interact with the C-terminal residues 1490 to 2108 of NOT1 in no particular order. NOT4 is placed on the periphery of NOT2 and NOT5, and it is presumed that NOT3 makes contacts with both NOT2, NOT5, or NOT4 and the N terminus of NOT1.

NOT3 or LexA-NOT5 was immunoprecipitated. These results indicate that it is the C-terminal portion of NOT1 protein (residues 1490 to 2108) that binds NOT2, NOT4, and NOT5. These data establish a clear physical separation of CCR4 and CAF1 from NOT2, NOT4, and NOT5 through their binding to separate regions of NOT1. Other limited interactions between these two groups of proteins can not be excluded, however.

Our data further show that NOT2 and NOT5 are closely linked. First, deletion of either of these components tends to result in decreased abundance of several other components in the complex. NOT2 and NOT5 appear to have general effects on the integrity or stability of the CCR4-NOT complex. Second, both proteins interacted in the two-hybrid system with the C-terminal segment of NOT1 and immunoprecipitated with the same segment of NOT1. Third, NOT3, NOT4, CAF1, or CCR4 defects did not affect the ability of LexA-NOT5 to immunoprecipitate NOT2. Fourth, NOT5 antibody coimmunoprecipitated NOT2, some NOT1, and no other CCR4-NOT component. These data implicate a close physical association between NOT2 and NOT5 that is important to the stability of the CCR4-NOT complex.

While NOT3 and NOT4 appear to be peripheral to NOT2 and NOT5, the location of the NOT3 protein could not be readily determined. For one thing, in all mutant strains that we analyzed by gel filtration analysis, NOT3 migrated at or near its monomeric size. NOT3 appears to be less stably associated with the CCR4-NOT complex. Immunoprecipitating CCR4 and CAF1 showed that NOT3 failed to coimmunoprecipitate in a *not1-2* strain, suggesting NOT3 associated with the C terminus of NOT1. However, when LexA-NOT1(1–1152) was immunoprecipitated, a small and reproducible amount of NOT3 was coimmunoprecipitated. No NOT3 was observed to coimmunoprecipitate with LexA-NOT1(1490–2108). Also, NOT3 did not require NOT4 for association with NOT1, CCR4, or CAF1. Since neither a *not3* nor a *not4* deletion affected the ability of LexA-NOT5 to immunoprecipitate NOT1, CCR4, CAF1, or NOT2, it appears that NOT3 and NOT4 are peripheral to NOT2 and NOT5. However, a *not3* deletion did reduce the ability of LexA-NOT5 to immunoprecipitate CCR4. In Fig. 7, we therefore assign NOT3 a place that includes contacts to the N terminus of NOT1 and binding to the outskirts of NOT2 and NOT5.

In our model for the physical arrangement of the components of the CCR4-NOT complex based on the above data (Fig. 7), CCR4 associates at one end of the complex through binding CAF1, which in turn binds the central portion of NOT1 (residues 667 to 1152). NOT2 and NOT5 bind the

C-terminal region of NOT1 (residues 1490 to 2108). NOT4 is on the outside of NOT2 and NOT5. NOT3 may display multiple contacts both to the N terminus of NOT1 and to NOT2 and NOT5.

CCR4 and CAF1 differ phenotypically from NOT2 to NOT5. The above biochemical data define CCR4 and CAF1 as a separate group of proteins within the CCR4-NOT complex that contacts the NOT1 protein in a distinct location from the NOT2, NOT4, and NOT5 proteins. Based on these identified separate locations within the complex, we would expect these subgroups of proteins to exhibit specific differences in both function and the proteins with which they interact. Phenotypically, CCR4 and CAF1 can display a number of functions that are different from those displayed by the other NOT proteins. This distinction correlates with the above-defined physical interactions. First, the NOT1 protein [NOT1(1490–2108)] which lacks the binding site for CAF1 and CCR4 is defective in *ADH2* derepression. Adding back to yeast in *trans* the N-terminal segment of NOT1 (residues 1 to 1152) can rescue this defect in *ADH2* expression, implicating the binding of CAF1 and CCR4 to this region as important for full *ADH2* expression. Second, the *not4* and *not5* disruptions augment *ADR1-5^C*-induced *ADH2* expression under glucose growth conditions, whereas *caf1* has no effect and *ccr4* causes a reduction in expression. Third, at other promoters such as *HO-lacZ* and *FKS1-lacZ*, the *not1*, *not2*, and *not4* defects (*not5* was not tested) result in increased expression whereas *caf1* and *ccr4* result in defects in expression (20). Fourth, the *not* alleles were identified in a genetic screen for increased 3-AT resistance. In contrast, little or no effect on 3-AT resistance is observed for *caf1* or *ccr4* defects (20). Fifth, whereas *not* alleles cause increased 3-AT resistance, *ccr4* and *caf1* defects can suppress these effects.

In addition, the *ccr4* and *caf1* defects when combined with *not* alleles resulted in synthetic phenotypes. *ccr4* or *caf1* were lethal with either *not2* or *not5* deletions, whereas *ccr4 caf1* and *not2 not5* double knockouts are viable. Although other explanations are possible with respect to synthetic growth defects, the synthetic defect of *ccr4* and *caf1* with that of *not2* and *not5* is consistent with the idea that these two groups of proteins display separate functions that are important for the overall integrity and activity of the CCR4-NOT complex. It is also possible that NOT2 and NOT5 display functions redundant with those of CCR4 and CAF1, although we feel that this is unlikely because of their dissimilarity in protein sequence and their actual physical separation and distinctiveness within the CCR4-NOT complex. Similarly, *ccr4 not1-2* and *caf1 not1-2* knockouts also displayed synthetic phenotypes. *not1-2* results from a stop codon (26) that causes 90% of the NOT1 protein to be about 1,100 amino acids long, which confirms that loss of the C terminus of the not1-2 protein in combination with *ccr4* or *caf1* defects results in a synthetic phenotype. The nonlethality of this combination is most likely due to the existence of some full-length NOT1 protein. These data also suggest that the lethality that results from deleting the *not1* gene may be caused by the combined loss of essential parts of the CCR4-CAF1 components and the NOT2 to NOT5 components. NOT1 may, therefore, be an essential protein due to its structural role in forming and maintaining the CCR4-NOT complex. However, the C-terminal region of NOT1 (residues 1490 to 2108) encompassing the site of binding NOT2, -4, and -5 by itself can complement a *not1* disruption, unlike the region of NOT1 binding CCR4 and CAF1 (residues 667 to 1152). The C terminus of NOT1 may be essential due to its ability to bind multiple NOT proteins, and/or the C terminus of NOT1 conveys another, as yet undetermined essential function.

It should also be noted that *not4* or *not3* deletions in combination with *ccr4* or *caf1* did not result in synthetic phenotypes. Therefore, although NOT2 and NOT5 appear important to the integrity of the complex, the lethality between *not2* or *not5* and that of *ccr4* or *caf1* is not simply due to loss of structural roles for NOT2 and NOT5 and their presumed importance for binding NOT3 and NOT4. Instead, NOT2 and NOT5 must play an important biochemical role independent of their mere physical presence in the CCR4-NOT complex. The observation that *not3* and *not4* deletions do not display synthetic growth defects with that of *ccr4* and *caf1* suggest either that these proteins as a group are not required for the function of any essential genes or that they actually function in the same pathway. It should be noted, though, that the *not3-2* allele in combination with *ccr4* resulted in a synthetic growth defect, suggesting that the not3-2 protein affects a particular interaction in a negative manner that is worse than the complete loss of the NOT3 protein.

Roles of the 1.9- and 0.9-mDa CCR4-NOT complexes. CCR4, CAF1, NOT1, NOT3, and NOT5 have all been found to associate in 1.9- and 0.9-mDa complexes (reference 20 and results herein), and NOT2 has also been shown to migrate at 0.9-mDa (unpublished observation). The association of CCR4 in the 0.9-mDa complex requires CAF1 (20) and, as we have shown here, the N-terminal region of NOT1. A *caf1* deletion reduced significantly the ability of CCR4 to associate in the 1.9-mDa complex (20) but did not eliminate it entirely. CCR4 must, therefore, be able to interact in the 1.9-mDa complex independently of CAF1 or its association with NOT1. The contacts and function for CCR4 in the 1.9-mDa complex may be separable from or additional to those it displays in the 0.9-mDa complex. Relatedly, CCR4 must express a function separate from its presence in the 0.9-mDa complex since a *ccr4* deletion was lethal when combined with the strain expressing only pNOT1(1490–2108). It remains possible that CCR4 exists in multiple 1.9-mDa complexes (3).

Other proteins that have been found to associate with CCR4, CAF1, or NOT proteins are DHH1 (15), CAF4, CAF16 (21), DBF2 (22), and MOB1 (18). These proteins may all be candidates for components of the 1.9-mDa complex. DHH1, CAF16, and MOB1 (3a) do not immunoprecipitate with CCR4 and are not components of the 0.9-mDa complex. However, CAF16 (21), DBF2, and MOB1 (unpublished observation) have been found to migrate in a 1.9-mDa complex. The complete components, assembly, and functional significance of the two CCR4-NOT complexes remain to be clarified. Characterizing the 1.9-mDa complex, understanding how the CCR4-NOT complexes interact with other transcriptional factors, and defining the particular roles of CCR4 and CAF1 in contrast to NOT2, NOT5, NOT3, and NOT4 should lead to a better conception of how the CCR4-NOT proteins function in both activated and repressed transcription.

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