The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively

Hai-Yan Liu, Vasudeo Badarinarayana, Deborah C.Audino, Juri Rappsilber[1], Matthias Mann[1] and Clyde L.Denis[2]

Department of Biochemistry and Molecular Biology, Rudman Hall, University of New Hampshire, Durham, NH 03824, USA and [1]Peptide and Protein Group, European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 10.2209, 69012 Heidelberg, Germany

[2]Corresponding author
e-mail: cldenis@christa.unh.edu

The CCR4 transcriptional regulatory complex consisting of CCR4, CAF1, DBF2 and other unidentified factors is one of several groups of proteins that affect gene expression. Using mass spectrometry, we have identified the 195, 185 and 116 kDa species which are part of the CCR4 complex. The 195 and 185 kDa proteins were found to be NOT1 and the 116 kDa species was identical to NOT3. NOT1, 2, 3 and 4 proteins are part of a regulatory complex that negatively affects transcription. All four NOT proteins were found to co-immunoprecipitate with CCR4 and CAF1, and NOT1 co-purified with CCR4 and CAF1 through three chromatographic steps in a complex estimated to be 1.2×10^6 Da in size. Mutations in the *NOT* genes affected many of the same genes and processes that are affected by defects in the CCR4 complex components, including reduction in *ADH2* derepression, defective cell wall integrity and increased sensitivity to mono- and divalent ions. Similarly, *ccr4*, *caf1* and *dbf2* alleles negatively regulated *FUS1–lacZ* expression, as do defects in the *NOT* genes. These results indicate that the NOT proteins are physically and functionally part of the CCR4 complex which forms a unique and novel complex that affects transcription both positively and negatively.

Keywords: activation/CCR4/NOT proteins/repression/transcription

Introduction

There are a number of general regulatory complexes that are involved in transcriptional processes. For example, in addition to the yeast holoenzyme that contains the SRB proteins (Wilson *et al.*, 1996), the SPT3–ADA2–GCN5 complex (Grant *et al.*, 1997), the NOT complex (Collart and Struhl, 1994), the PAF1 holoenzyme (Wade *et al.*, 1996) and the CCR4 complex have all been identified as playing roles in affecting gene transcription. Each of these groups of proteins appears to be unique. The interaction and functional relationship of these groups of transcriptional regulatory factors, however, remain to be clearly established. In this study, we demonstrate that the NOT protein complex is part of the CCR4 transcriptional

complex and that these two groups of proteins share overlapping functions.

CCR4 affects the expression of many genes and processes in yeast. It is required for the expression of *ADH2* and other non-fermentative genes (Denis, 1984; Denis and Malvar, 1990) and for unidentified genes involved in cell wall integrity (Liu *et al.*, 1997). *ccr4* mutations result in a partial cell cycle block during telophase and increase the sensitivity of yeast cells to Li$^+$ and Mg^{2+} (Liu *et al.*, 1997). *ccr4* is also a suppressor of *spt10* mutations (Denis, 1984), defects which result in enhanced transcription at *ADH2* (Denis and Malvar, 1990) and other loci (Natsoulis *et al.*, 1991). In addition to acting as an activator, CCR4 has been implicated in negatively affecting gene expression as well (McKenzie *et al.*, 1993; Schild, 1995). CCR4 is a component of a multi-subunit complex (Draper *et al.*, 1994). Two of the CCR4 complex components, CAF1 (POP2) (Sakai *et al.*, 1992; Draper *et al.*, 1995), and DBF2, a cell cycle-regulated protein kinase (Toyn *et al.*, 1991), function to control many of the same processes as CCR4 (Liu *et al.*, 1997). While none of these genes by themselves are essential, the phenotypes conferred by the *ccr4*, *caf1* and *dbf2* mutations indicate that the CCR4 complex is required for optimal and proper expression of many genes. The evolutionary conservation of CAF1 across eucaryotes (Draper *et al.*, 1995) further suggests that this complex plays an important role in eucaryotic gene control. Although the mechanism of how CCR4 functions remains unclear, the site of CCR4 action at the *ADH2* locus has been shown to occur at a post-chromatin remodeling step (Verdone *et al.*, 1997).

In addition to CAF1 and DBF2, the CCR4 complex contains several unidentified proteins, 195, 185, 140 and 116 kDa in size (Draper *et al.*, 1994). Our initial attempt at cloning the corresponding genes for these proteins by two-hybrid analysis was unsuccessful (Draper *et al.*, 1995; Liu *et al.*, 1997). Mass spectrometry has recently become the method of choice for rapid and unambiguous identification of gel-separated proteins. Large-scale analysis of yeast proteins is now possible (Shevchenko *et al.*, 1996), and entire yeast protein complexes can be studied (Lamond and Mann, 1997; Neubauer *et al.*, 1997). Here, we have used these methods to identify the 195, 185 and 116 kDa species of the CCR4 complex. The 185 and 195 kDa species were found to be NOT1 and the 116 kDa species was found to be NOT3.

The *NOT* genes have been identified as encoding a group of factors involved in repressing the transcription of *HIS3* from a non-canonical TATA (Collart and Struhl, 1994). This group of proteins contains NOT1/CDC39, NOT2/CDC36, NOT3 and NOT4/MOT2/SIG1, and genetic evidence indicates that they function as a complex *in vivo* (Collart and Struhl, 1993, 1994). In addition to affecting *HIS3* expression, the *not* mutations augment the
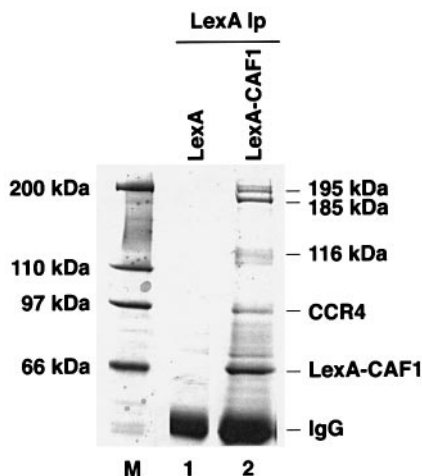
Fig. 1. Immunoprecipitation of the CCR4 complex for protein sequencing by mass spectrometry. The yeast whole cell extracts containing either LexA alone or full-length LexA–CAF1 were treated with the LexA antibody, and the resulting immunoprecipitates were subjected to SDS–PAGE. The resulting gel was stained with Coomassie blue. 'M' indicates the molecular weight standard. Lanes 1 and 2 are the immunoprecipitates from extracts containing LexA alone and LexA–CAF1, respectively. The 195, 185 and 116 kDa species in lane 2 were excised prior to mass spectrometric analysis.

expression of many genes or reporter genes, confirming their role as a repression complex (Cade and Errede, 1994; Collart and Struhl, 1994; Irie *et al.*, 1994; Collart, 1996). Of the four *NOT* genes, only *NOT1* was found to be essential. We have subsequently shown that NOT2 and NOT4 also associate with the CCR4 complex. Genetic analyses reveal that *NOT* defects result in phenotypes similar to those observed with the deletion of *CCR4* and its associated components. These results indicate that the CCR4 complex includes the NOT proteins and that this complex can affect gene transcription both positively and negatively.

Results

The 185/195 and 116 kDa proteins in the CCR4 complex are NOT1 and NOT3

To identify the proteins which associate with CCR4, the CCR4 complex was isolated by immunoprecipitation. Yeast extracts, containing either a LexA–CAF1 fusion protein or just LexA alone, were incubated with an antibody directed against the LexA protein, and the resulting immunoprecipitates were subjected to SDS–PAGE (Figure 1). After staining the proteins, the 116, 185 and 195 kDa species that specifically co-immunoprecipitated with CCR4 (Draper *et al.*, 1994) were isolated and were analyzed by mass spectrometry using the strategy previously described (Shevchenko *et al.*, 1996). A small aliquot of the peptide mixture resulting from in-gel digestion of the bands was analyzed by matrix-assisted laser desorption/ionization (MALDI). High resolution peptide mass maps were obtained of all three bands which were analyzed. Database searches with the set of measured masses resulted in the following identifications: band 116 kDa was NOT3, band 185 kDa was NOT1 and band 195 kDa was also NOT1. The identification of NOT3 was performed by MALDI peptide mapping only. The database

search revealed that 26 measured peptide masses fit the sequence of NOT3 within a mass accuracy of 50 p.p.m. This corresponds to 30% of the sequence. The other two bands were subjected to both MALDI peptide mapping and mass spectrometric sequencing using nanoelectospray (Wilm *et al.*, 1996). The peptide maps covered 29% of the protein in the band migrating at 185 kDa and 32% of the protein in the band migrating at 195 kDa. The identification of the lower band is shown in Figure 2. Sequencing of 10 of the peptides derived from the 185 kDa band and eight of the peptides derived from the 195 kDa band confirmed the identification (data not shown). No peptides of the N-terminal region of the NOT1 protein were found in the analysis of the lower band. Thus, the data are consistent with the N-terminal truncation of the NOT1 protein suggested by previous studies (Collart, 1996).

NOT2 and NOT4 are also in the CCR4 complex

The NOT1 and NOT3 proteins have been shown to be part of a complex that also includes the NOT2 and NOT4 proteins (Collart and Struhl, 1994). To examine the possibility that the NOT2 and NOT4 proteins were also part of the CCR4 complex, we carried out a series of immunoprecipitation experiments. We first examined the association of NOT1 with CCR4. A LexA–NOT1 fusion was expressed in a wild-type strain. LexA–NOT1 was immunoprecipitated with the LexA antibody while the CCR4 complex was immunoprecipitated with the CCR4 antibody. The resulting immunoprecipitates were subjected to Western blot analysis (Figure 3A). CCR4 co-immuno-precipitated with LexA–NOT1 (Figure 3A, lane 3) while LexA–NOT1 along with the NOT1 proteins (185/195 kDa) were co-immunoprecipitated with CCR4 (Figure 3A, lane 5). These results confirm the protein sequencing data.

To investigate the association of NOT2 with the CCR4 complex, a LexA–NOT2 fusion was expressed in a wild-type strain, a *ccr4Δ* strain and a *caf1Δ* strain. An antibody raised against the LexA protein was used to immunopre-cipitate the LexA–NOT2 fusion while antibodies raised against either CCR4 or CAF1 were used to bring down CCR4 and CAF1, respectively. The resulting immunopre-cipitates were subjected to SDS–PAGE, followed by Western blot analysis (Figure 3A and B). Immunoprecipit-ating LexA–NOT2 with the LexA antibody resulted in co-immunoprecipitation of NOT1 from the wild-type, *ccr4Δ* and *caf1Δ* extracts (Figure 3A, lane 4, and B, lanes 1 and 2, respectively). CCR4 co-immunoprecipitated along with LexA–NOT2 and NOT1 from the wild-type strain (Figure 3A, lane 4), but not from the *caf1Δ* strain (Figure 3B, lane 1). When the CCR4 antibody was used to repeat the immunoprecipitation experiments, the NOT1 and LexA–NOT2 proteins were found to co-immuno-precipitate with CCR4 from the wild-type strain (Figure 3A, lane 6), but not from the strains lacking either CAF1 (Figure 3B, lane 3) or CCR4 (Figure 3B, lane 4). Longer exposures of the results presented in Figure 3B, lane 3, indicated that a small amount of NOT1 and LexA–NOT2 was found to co-immunoprecipitate with CCR4 from the *caf1Δ* strain (data not shown). These results indicate that NOT2 physically interacts with both CCR4 and NOT1, and that the association of CCR4 with the NOT proteins is largely dependent on the presence of
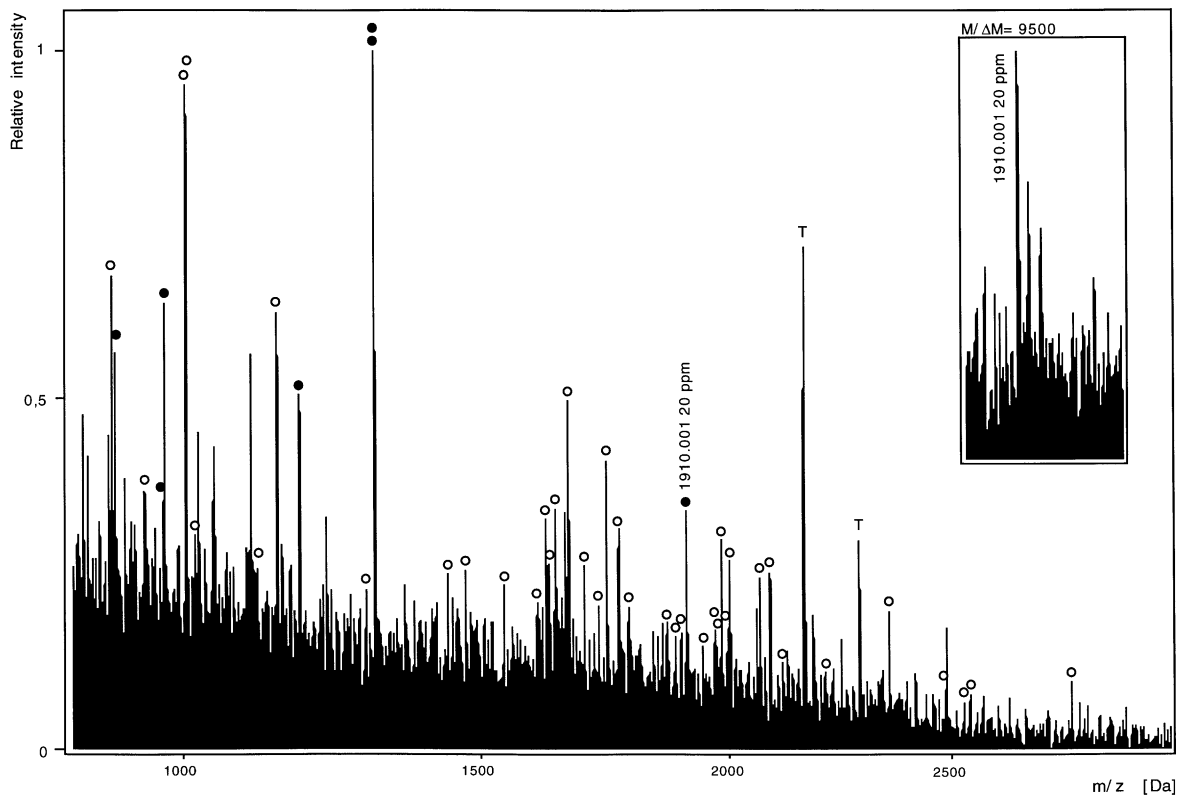
Fig. 2. Identification of the yeast protein NOT1 from the 185 kDa band by MALDI mass spectrometry. The figure shows the MALDI mass spectrum obtained after in-gel digestion of the 185 kDa band. Ion signals whose measured masses match calculated masses of tryptic peptides of NOT1 within 50 p.p.m. are indicated with circles. Filled circles mark those ion signals whose corresponding peptides were sequenced additionally by nanoelectrospray mass spectrometry. In one case, nanoelectrospray sequencing revealed two peptides for one measured peptide mass (peak at 1183.634 Da, marked by two filled circles). Ion signals corresponding to trypsin autolysis products are labeled with the letter 'T'.

CAF1. The immunoprecipitation experiments were also repeated by using the CAF1 antibody. NOT1, LexA–NOT2 and CCR4 were found to co-immunoprecipitate with CAF1 from the wild-type strain (data not shown), and NOT1 and LexA–NOT2 were co-immunoprecipitated with CAF1 from the *ccr4Δ* strain (Figure 3B, lane 6). However, NOT1 and LexA–NOT2 failed to co-immuno-precipitate with the CAF1 antibody from the *caf1Δ* strain (Figure 4B, lane 7), confirming that LexA–NOT2 does not immunoprecipitate fortuitously with the CAF1 antibody. These results also suggest that the interaction between CAF1 and the NOT proteins is CCR4 independent.

To address the question as to whether NOT4 was in the CCR4 complex, a c-Myc-tagged NOT4 fusion was expressed along with LexA–CAF1 in a wild-type strain. Extracts treated with the LexA antibody resulted in co-immunoprecipitation of c-Myc–NOT4 with LexA–CAF1, CCR4 and NOT1 (Figure 4A, lane 3), while the LexA pre-immune serum failed to immunoprecipitate these proteins (Figure 4A, lane 2). The c-Myc–NOT4 protein also co-immunoprecipitated with CCR4 and NOT1 when the extracts were immunoprecipitated with either CAF1 anti-body (Figure 4A, lane 4) or CCR4 antibody (Figure 4A, lane 5). Immunoprecipitation with the c-Myc antibody, in turn, was able to bring down LexA–CAF1, CCR4 and NOT1 along with c-Myc–NOT4 (lane 6). We also immuno-precipitated the CCR4 complex from an extract prepared from a strain expressing both LexA–NOT2 and c-Myc–NOT4 fusion proteins. The resulting immunoprecipitates were analyzed by Western blot (Figure 4B). It is clear that

NOT1, NOT2 and NOT4 co-immunoprecipitated with CCR4 and CAF1. Because NOT3 is also in the CCR4 complex as determined by mass spectrometry, we conclude that the complete NOT repressive regulatory complex is part of the CCR4 complex.

Two-hybrid analysis was used further to examine the interaction of the NOT proteins and the CCR4 complex components. As shown in Table I, both B42–NOT1 and B42–NOT2 interacted with LexA–CAF1, and LexA–NOT1 was found to interact with B42–CAF1. LexA–CCR4 interacted with B42–NOT1, and B42–DBF2 inter-acted well with LexA–NOT2. The multiplicity of these interactions confirms the above-described protein analyses.

The CCR4 complex is a unique transcriptional regulatory complex

Our previous studies on CCR4 indicated that the CCR4 complex is a transcriptional regulatory complex distinct from that of several other complexes such as the SNF/SWI complex, the yeast holoenzyme and the putative SPT4, 5, 6 complex (Denis *et al.*, 1994). The size of the CCR4 complex was estimated following Superose 6 gel filtration chromatography. As shown in Figure 5A, CCR4 migrated in two separate peaks of 1.9×10^6 and 1.0×10^6 Da. In other experiments, a small portion of CCR4 migrated at 2.0×10^5 Da, which is close to the size of CCR4 and may represent monomeric CCR4 (Figure 5C, top panel). The two larger complexes were also unaffected by prior DNase treatment, suggesting that they do not result from non-specific binding to DNA (Figure 5A, data
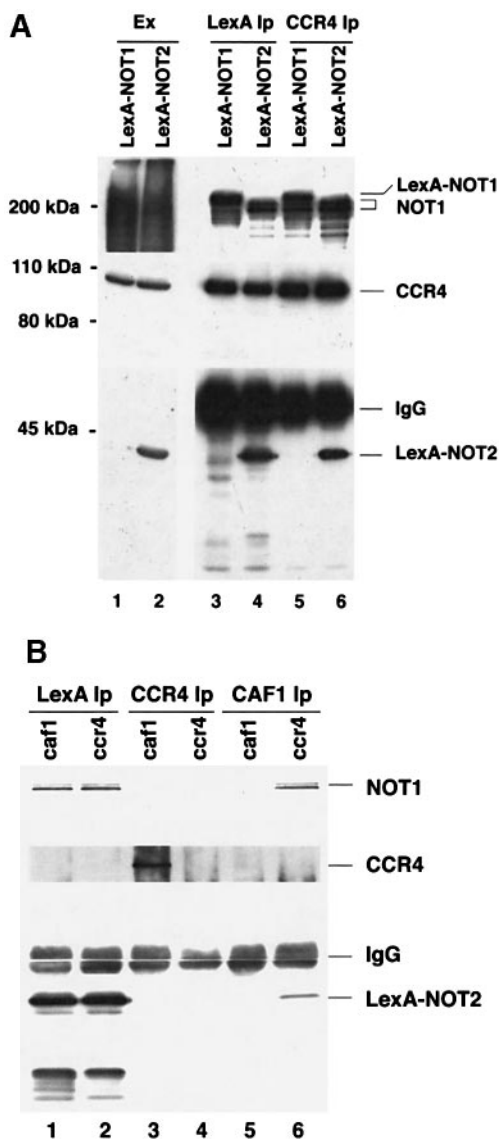
Fig. 3. Co-immunoprecipitation of the NOT1 and NOT2 proteins with the CCR4 complex. (**A**) The yeast whole cell extracts containing either LexA–NOT1 or LexA–NOT2 were treated with LexA antibody (lanes 3 and 4) or CCR4 antibody (lanes 5 and 6). The resulting immunoprecipitates along with the crude extracts (lane 1 and 2) were subjected to immunoblot analysis and probed with NOT1, CCR4 and LexA antibodies. (**B**) The yeast whole extracts containing LexA–NOT2 prepared from a *caf1*-deleted strain (lanes 1, 3 and 5) or a *ccr4*-deleted strain (lanes 2, 4 and 6) were treated with LexA antibody (lanes 1 and 2), CCR4 antibody (lanes 3 and 4) or CAF1 antibody (lanes 5 and 6). The resulting immunoprecipitates were subjected to immunoblot analysis and probed with NOT1, CCR4 and LexA antibodies. The bands beneath LexA–NOT2 in lanes 1 and 2 represent degradation products of LexA–NOT2 (data not shown).

not shown). The 1.9×10^6 Da CCR4 complex is separate from that of the SRB complex which, as analyzed on a longer Superose 6 column, migrated at 1.7×10^6 Da (Figure 5B). Moreover, in a *caf1Δ* strain, most of the CCR4 protein was found at the 1.0×10^5 Da size, indicating that the CAF1 protein is required for CCR4 association in the 1.9×10^6 and 1.0×10^6 Da complexes (Figure 5C, top two panels). A *caf1Δ* had no effect, however, on the ability of the non-CCR4 complex component, SPT10, to migrate at 1.9×10^6 Da (Figure 5C, bottom two panels),
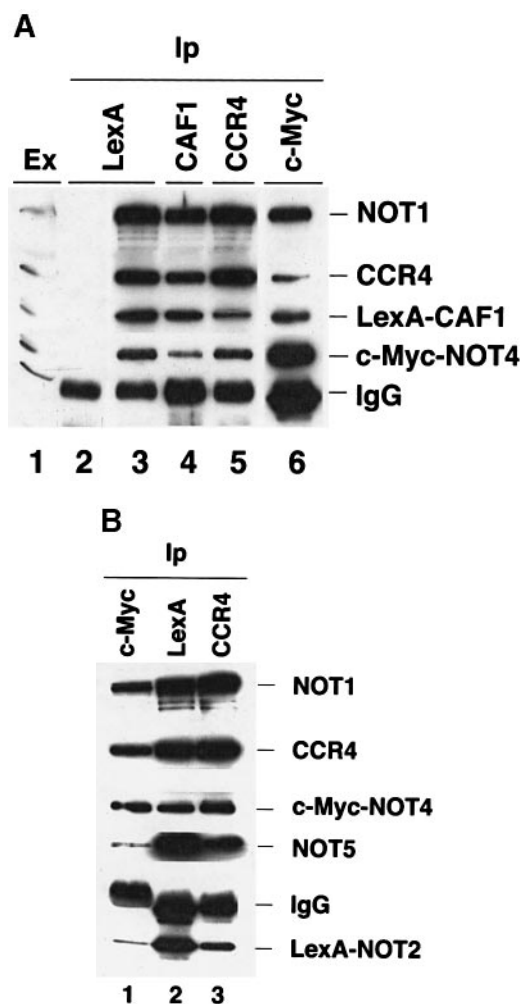
Fig. 4. Co-immunoprecipitation of the NOT1, 2 and 4 proteins with the CCR4 complex. (**A**) Yeast whole cell extracts containing c-Myc–NOT4 and LexA–CAF1 were treated with LexA pre-immune serum (lane 2), LexA antibody (lane 3), CAF1 antibody (lane 4), CCR4 antibody (lane 5) or c-Myc antibody (lane 6). The resulting immunoprecipitates along with the crude extract (lane 1) were subjected to immunoblot analysis and probed with NOT1, CCR4, LexA and c-Myc antibodies. (**B**) Yeast whole cell extracts containing LexA–NOT2 and c-Myc–NOT4 were treated with c-Myc antibody (lane 1), LexA antibody (lane 2) or CCR4 antibody (lane 3). The resulting immunoprecipitates were subjected to immunoblot analysis and probed with NOT1, CCR4, c-Myc, NOT5 and LexA antibodies.

Table I. Two-hybrid interaction assay

	β-Gal activity (U/mg)				
	B42–NOT1	B42–NOT2	B42–CAF1	B42–DBF2	B42
LexA–NOT1	–	–	130	–	3.6
LexA–NOT2	–	900	270	660	110
LexA–CCR4	330	–	1100	74	6.4
LexA–CAF1	1100	380	–	930	86
LexA	<2	<2	<2	<2	<2

LexA–CCR4, –NOT1 and –NOT2 contain full-length CCR4, NOT1 and NOT2. LexA–CAF1 contains residue 127–444 of CAF1. All LexA fusions contain residues 1–202 of LexA. B42–NOT1, –NOT2 and –DBF2 contain full-length NOT1, NOT2 and DBF2. B42–CAF1 contains residues 148–444 of CAF1. – indicates the β-galactosidase activity is no greater than the background interaction with B42 alone.
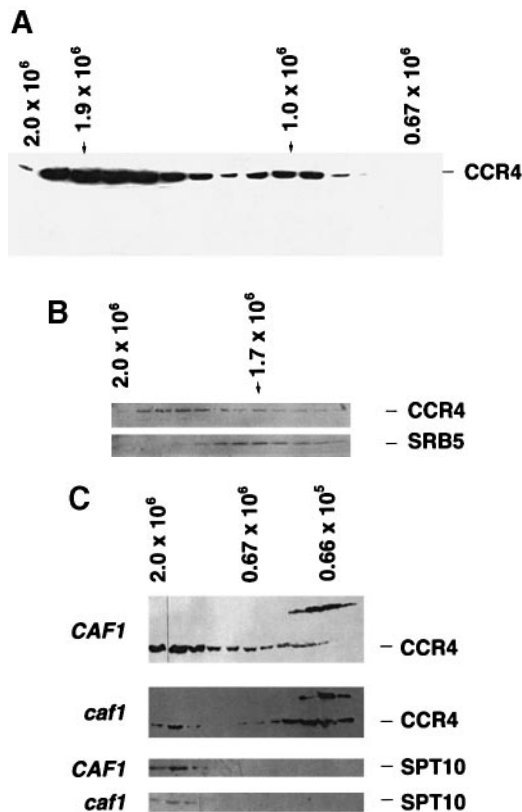
Fig. 6. Co-purification of the NOT1 protein and the CCR4 complex. Yeast whole cell extracts prepared from a *caf1*-deleted strain containing *CAF1–6His* integrated at the *TRP1* locus were chromatographed on a Ni^{2+}-NTA, Mono Q and Superose 6 HR10/30 column as described in Materials and methods. Fractions of 0.5 ml from the Superose 6 chromatography were subjected to immunoblot analysis and probed for NOT1, CCR4 and CAF1. The Mono Q lane refers to the peak fraction following Mono Q chromatography that was applied to the Superose 6 column. The Ni^{2+}-NTA eluate was not probed for NOT1. The arrow indicates the size of the peak eluted from the Superose 6 column that contains NOT1, CCR4 and CAF1–6His.

Fig. 5. Analysis of the CCR4 complexes using gel filtration chromatography. (**A**) Yeast whole cell extracts prepared from a wild-type strain and treated with DNase were chromatographed on a Superose 6 HR10/30 column. The resulting 1 ml fractions (30 µl of each fraction) were subjected to immunoblot analysis and probed with CCR4 antibody. The two arrows indicate the size of the two peaks containing CCR4. (**B**) Yeast whole cell extracts were chromatographed on an extended Superose 6 HR16/50 column. The resulting 1 ml fractions (first 12 fractions) were subjected to immunoblot analysis and probed with CCR4 and SRB5 antibodies. The arrow indicates the peak containing SRB5. (**C**) Yeast whole cell extracts prepared from a wild-type or a *caf1*-deleted strain were chromatographed on the Superose 6 HR10/30 column. The resulting 1 ml fractions (every other fraction is displayed) were subjected to immunoblot analysis and probed for CCR4 (top two panels) and SPT10 (bottom two panels). '*CAF1*' and '*caf1*' indicate the wild-type and *caf1*-deleted strains, respectively. The 120 kDa band that runs above CCR4 in the CCR4-probed panel represents a non-specific protein and serves as an internal control for the experiment. Based on this control, the amount of protein loaded for the '*CAF1*' experiment was about twice that of the '*caf1*' experiment, resulting in the decreased level of CCR4 protein visible in the '*caf1*'experiment for the SPT10 Western (bottom panel).

nor on the SRB5 protein to migrate at 1.7×10^6 Da (data not shown).

To analyze the CCR4 complex further, we isolated the CCR4 complex from a strain in which the *CAF1* gene was deleted and a *CAF1* gene tagged at its C-terminus with 6×His was integrated into the genome at the *TRP1* locus. This *CAF1–6His* gene was able to complement the defect of *caf1*Δ (Liu *et al.*, 1997). The extracts prepared from this strain were first put onto a Ni^{2+}-NTA column, and the bound proteins was eluted with 250 mM imidazole. The NOT1 protein and CAF1–6His were found to co-immunoprecipitate with CCR4 when the Ni^{2+} eluate was treated with CCR4 antibody (data not shown). The Ni^{2+} eluate subsequently was loaded onto a Mono Q column, and the bound proteins were eluted in a linear salt gradient.
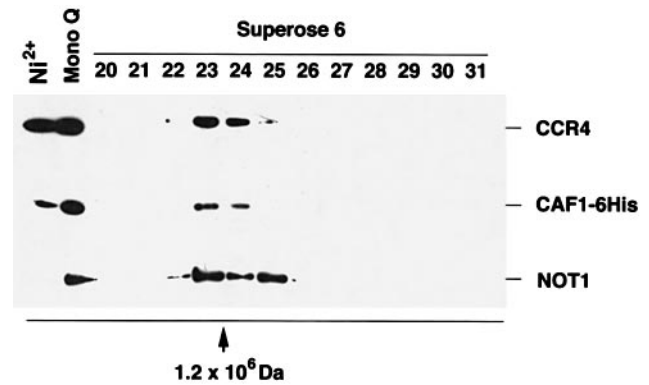
The Mono Q fractions were analyzed by Western blot using both CCR4 and CAF1 antibody, and CCR4 and CAF1–6His were found to co-elute (Liu *et al.*, 1997; Figure 6). Fractions containing both CCR4 and CAF1 were pooled and the proteins were analyzed further by Superose 6 gel filtration chromatography. The fractions from these different steps in purification were subjected to Western blot analysis. The purified CCR4 complex displayed a molecular weight of 1.2×10^6 Da following the Superose 6 gel filtration chromatography (Figure 6), corresponding closely to the 1.0×10^6 Da CCR4 complex observed in crude extracts (Figure 5A). NOT1, CCR4 and CAF1 were all found to co-purify through these three purification steps. In contrast, Western blot analysis using antibodies against SRB5 and SRB6 failed to detect either of these proteins in the Mono Q and Superose 6 fractions (data not shown). These data indicate that NOT1, CCR4 and CAF1 are components of the same complex. In addition, the 1.9×10^6 and 1.2×10^6 Da CCR4 complexes appear distinct from the yeast holoenzyme containing the SRB complex.

Mutations in the NOT genes result in similar phenotypes to those observed with ccr4 and caf1 alleles

The presence of the NOT proteins in the CCR4 complex suggest that they should function to control similar genes and processes as do CCR4 and its associated components. However, the NOT proteins have been characterized as a repression complex and CCR4 is generally considered to be an activator. To address this issue, we analyzed the effect of *not* mutations on several processes known to be affected by *ccr4*. The results from the phenotypic analyses are summarized in Table II. Mutations in the *NOT* genes except for *NOT3* reduced *ADH2* expression under non-fermentative conditions, indicating that the NOT proteins can act as activators. A *not4* allele was also capable of suppressing the enhanced *ADH2* expression that is caused

Table II. Phenotypic analysis

Strains	ADH II	*spt10* ADH II	Caffeine 8 mM	37°C YD	37°C YD 1 M sorbitol	Mg^{2+} 750 mM	Stauro 1 mg/ml	3 AT 20 mM
wt	2400	91	+	+	+	+	+	–
ccr4	400	23	–	+	+	–	–	–
caf1	1000	7	w	–	+	–	–	w/–
not1	1300	78	–	w	w	–	–	+
not2	340	86	–	–	+	–	w	+
not3	2500	N.D.	+	+	+	+	w	+
not4	1200	13	w	–	w	w	–	+

Growth was scored on YD plates as supplemented with 8 mM caffeine, 1 mg/ml of staurosporine (stauro), 750 mM MgCl$_2$ or 1 M sorbitol as indicated. 3AT: growth was scored on minimal plates lacking histidine and containing 20 mM 3-aminotriazole (3AT) using strains isogenic to KY803 (wt) containing the YCp88-Sc4363 plasmid (Collart and Struhl, 1994). Strains used for monitoring caffeine, Mg^{2+}, temperature and staurosporine sensitivity were KY803 (wt), EGY188-1 (*ccr4*), EGY188-c1 (*caf1*), MY8 (*not1*), MY16 (*not2*), MY508 (*not3*) and MY537 (*not4*). Wild-type strain EGY188 gave the same results as KY803. ADH II activities (mU/mg) represent the average of at least three determinations and were conducted following growth at 30°C on YEP medium containing 3% ethanol. No effect was observed in the *not* mutations on ADH II activity under glucose growth conditions (data not shown). The SEM for the ADH II activities was <20%. For ADH II assays, the following strains were used: wt, KY803-Δ3; *not1*, MY8-Δ1; *not2*, MY16-Δ1; *not3*, MY25-Δ1; *not4*, 612-1d-n4; and for *spt10* ADH II assays the strains were: wt, *spt10* segregants from cross 808-5c and 612-1d-n4; *not1*, *spt10 not1-2* segregants from cross MY8Δ1 and 1366-4a; *not2*, *spt10 not2* segregants from cross 808-5c and MY16-Δ1; *not4*, *spt10 not4* segregants from cross 808-5c and 612-1d-n4. The isogenic parent for 612-1d-n4 is 612-1d whose ADH II activity is 3000 mU/mg. ADH II and *spt10* ADH II activities for *ccr4* and *caf1* strains are taken from Denis (1984) and Draper *et al.* (1995). N.D., not done; '+', good growth; 'w', weak growth; '–', no or poor growth.

by an *spt10* defect. All of the *not* alleles except for *not3* also displayed sensitivity to caffeine, a phenotype resulting from defects in cell wall integrity, which is shared by the *ccr4*, *caf1* and *dbf2* alleles (Liu *et al.*, 1997). *ccr4*, *caf1* and *dbf2* mutations also result in temperature- and/or cold-sensitive phenotypes that are suppressible by 1 M sorbitol, confirming their roles in control of cell wall integrity (Liu *et al.*, 1997). In agreement with this phenotype, it has been shown previously that a *not4* allele confers a temperature-sensitive phenotype that is suppressible by 1 M sorbitol (Cade and Errede, 1994). We subsequently found that the *not2* ts phenotype was also relieved by 1 M sorbitol (Table II). Also, the caffeine-sensitive phenotype of *not4* was suppressed by 1 M sorbitol (data not shown). In agreement with these results, *not2*, *not3* and *not4* alleles were sensitive to staurosporine, an inhibitor of PKC1, indicative of cell wall defects. Moreover, *not1*, *not2* and *not4* alleles were sensitive to 0.04% SDS, another phenotype indicative of a defect in cell wall integrity (Igual *et al.*, 1996) also displayed by *ccr4*, *caf1* and *dbf2* alleles (data not shown). Furthermore, *not1*, *not2* and *not4* alleles were sensitive to high concentrations of the divalent cation, Mg^{2+}, as are *ccr4*-, *caf1*- and *dbf2*-containing strains (Table II). These results indicate that defects in the NOT factors result in phenotypes consistent with the NOT proteins functioning in processes similar to CCR4, CAF1 and DBF2.

The CCR4 complex has positive and negative effects on gene transcription

To address whether the CCR4 complex components can act as repressors in a manner similar to that observed for the NOT proteins, we examined the effect of *ccr4*, *caf1* and *dbf2* defects on *FUS1–lacZ* expression. Mutations in *NOT* genes result in increased expression of the *FUS1* gene or the *FUS1–lacZ* reporter gene in the absence of pheromone stimulation (Cade and Errede, 1994; Collart and Struhl, 1994; Irie *et al.*, 1994). As shown in Figure 7A, deletion of *CAF1* caused a 5-fold increase in β-galactosid-ase activity from the *FUS1–lacZ* reporter, while deletion of

CCR4 and *DBF2* resulted in an increase of β-galactosidase activity of ~2- and 3-fold, respectively. These results are similar to the 2- to 5-fold effects observed for the *not* effects on the *FUS1* promoter. The *ccr4*, *caf1* and *dbf2* effects on the *FUS1–lacZ* reporter were specific to the *FUS1* promoter since *ccr4*, *caf1* and *dbf2* had very different effects on other *lacZ* reporters carrying different promoters (see below).

To extend the comparison of the NOT proteins and the CCR4 complex components, we examined the effects of their mutations on several other reporter genes. All reporter genes, including the *FUS1–lacZ* reporter, are derived from a *UAS-less lacZ* reporter. As shown in Figure 7B, mutations in the *NOT* genes, *CCR4* and *CAF1* resulted in decreased expression of the *CYC1–lacZ* reporter gene (dependent on the HAP2, 3, 4 and 5 activator complex), in which the *ccr4*, *caf1* and *not2* alleles had the greatest effects. The effect on *CYC1–lacZ* expression was more severe when cells were supplied with a non-fermentable carbon source, such as ethanol and glycerol, than with glucose, but *ccr4*, *caf1* and *not3* also had effects under glucose growth conditions. The observed effects on the derepressed expression of the *CYC1–lacZ* reporter was not due to general effects on the plasmid or *lacZ* expression since the *FKS1–lacZ* reporter was largely unaffected under non-fermentative growth conditions by these same mutations (Figure 7C). These data confirm that, as observed with effects on *ADH2* expression, the *NOT* genes can also be involved in the activation of gene transcription.

Though the *NOT* genes and *CCR4* behave similarly, some variations in their effects on gene transcription were observed when we examined other *lacZ* reporter genes. In the case of *HO–lacZ* expression, *not1*, *not2*, *not4* and *dbf2* defects increased β-galactosidase activity while the strains containing deletion of *ccr4*, *caf1* or *not3* showed reduced β-galactosidase activity (Figure 7C). When the *FKS1–lacZ* reporter was examined, variation in the effects was again observed. The *not1*, *not4* and *dbf2* alleles caused 2-fold increases in β-galactosidase activity, while either *ccr4* or *not3* caused reductions in β-galactosidase activity
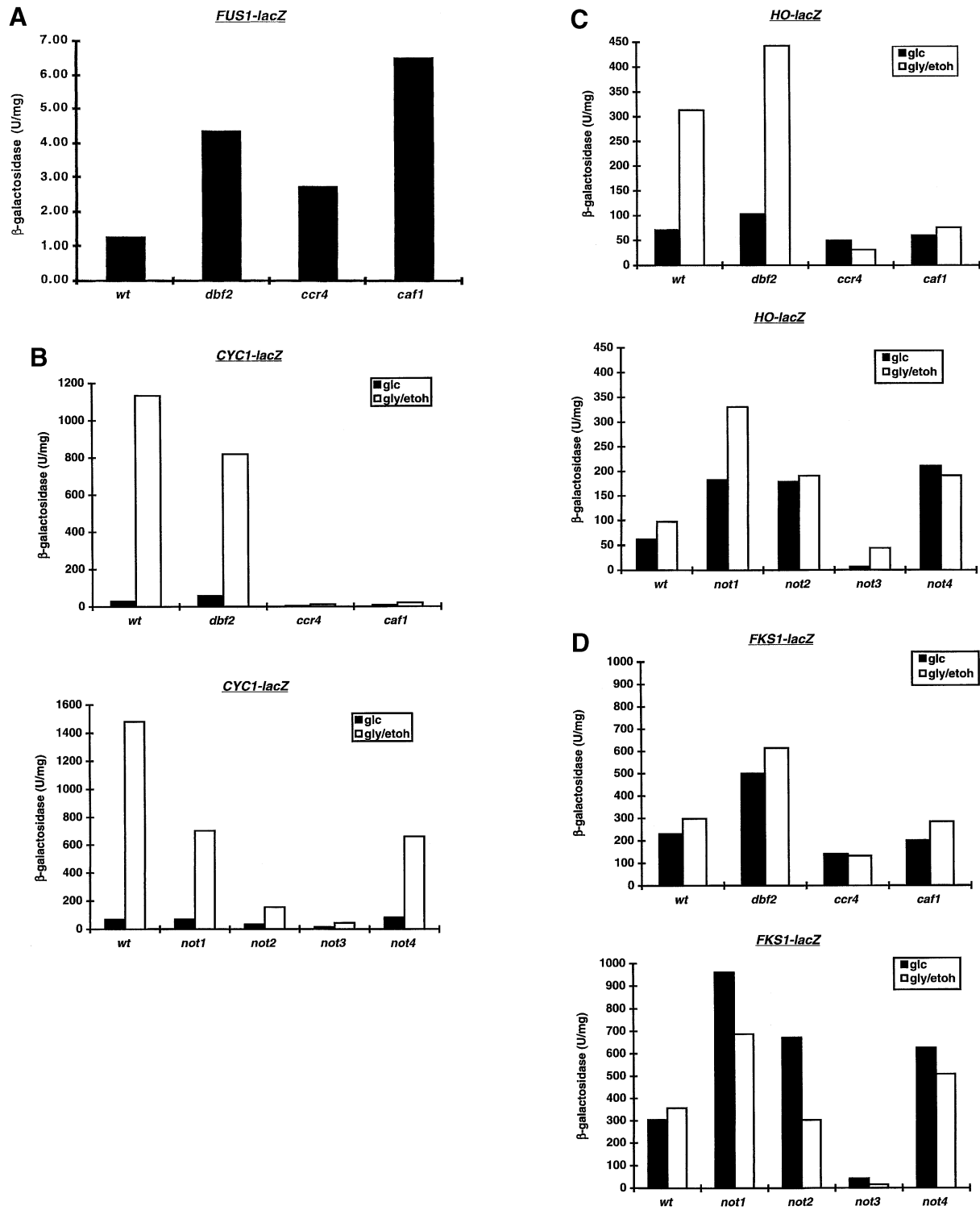
Fig. 7. Effects of the *ccr4*, *caf1*, *dbf2* and *not* mutations on regulation of gene expression. (**A**) β-Galactosidase activity in strains (grown on minimal medium lacking uracil and supplemented with 8% glucose) carrying a plasmid-borne *FUS1–lacZ* reporter gene without pheromone stimulation. Values are averages for at least five transformants, and the standard error of the mean (SEM) was <20% except for strain EGY188 in which it was 35%. wt, EGY188; *dbf2*, EGY188-d2; *ccr4*, EGY188-1-1; and *caf1*, EGY188-c1-1. (**B**) β-Galactosidase activity in strains carrying a plasmid-borne *CYC1–lacZ* reporter, LG265UP1, containing the upstream element for the HAP2, 3, 4 and 5 activator (Guarente and Mason, 1983). The upper panel displays the effects of *ccr4*, *caf1* and *dbf2* mutations on the expression of the *CYC1–lacZ* reporter while the lower panel gives the effects of the *not* mutations on expression of the same reporter. Values are averages for at least four transformants, and the SEM was <25% for glucose-grown culture whereas, for non-fermentative culture the SEM was <30%, except for strains EGY188-1-1 and EGY188-c1-1 in which it was <50%. Strains used for the upper panels for (B), (C) and (D) are the same as (A), and for the lower panels strains are: wt, KY803; *not1*, MY8; *not2*, MY16; *not3*, MY25; and *not4*, MY20. (C and D) The same experiments as (B) except that the *CYC1–lacZ* reporter was replaced by either a *HO–lacZ* reporter [containing the complete upstream sequence of the *HO* gene, plasmid BA161 (Breeden and Nasmyth, 1987)] (**C**) or a *FKS1–lacZ* reporter (plasmid pF712-380, Igual *et al.*, 1996) (**D**). Values are averages for at least three transformants, and the SEM was <25%.

(Figure 7D). The *not2*, and *caf1* alleles showed less dramatic or no effects on *FKS1–lacZ* expression. These results indicate that different components of the CCR4 complex have similar but not necessarily identical effects on gene expression.

We also analyzed the effect of *caf1* and *ccr4* defects on *HIS3* gene expression under conditions when the GCN4 activator is disabled. Using strain KY803, in which *not* mutations cause resistance to 20 mM 3-aminotriazole (3AT) whereas the parent strain is sensitive (Collart and Struhl, 1994) (Table II), we deleted *CCR4* and *CAF1*. A *ccr4* disruption in this strain background did not result in any enhanced *HIS3* expression and resistance to 20 mM 3AT whereas a *caf1* disruption resulted in weak growth at 20 mM 3AT, indicative of a slight increase in *HIS3* expression (Table II).

Discussion

The NOT negative regulatory complex is physically associated with the CCR4 transcriptional regulatory complex

Using mass spectrometry, we have identified the 195, 185 and 116 kDa species of the CCR4 complex. The 195 and 185 kDa species were found to be NOT1 and the 116 kDa species was found to be NOT3. The 185 kDa species is an apparent degradation product of NOT1 and is missing ~100 amino acids from the N-terminus. The 195 and 185 kDa species were also shown to react specifically with an antibody raised against a GST–NOT1 fusion protein. The NOT1 and NOT3 proteins are part of the NOT negative regulatory complex containing four proteins (NOT1, NOT2, NOT3 and NOT4) (Collart and Struhl, 1994). We subsequently showed by co-immunoprecipitation that the NOT2 and NOT4 proteins were also associated with the CCR4 complex. The interactions between the NOT proteins and the CCR4 complex were also confirmed by two-hybrid analysis (Table II). These results imply that there exist multiple interactions among these components and provide additional evidence that NOT proteins are part of the CCR4 complex. Recently, another component of the NOT complex, the NOT5 protein, has been found to be functionally and physically associated with the other NOT proteins (Oberholzer and Collart, 1998). We have since shown that NOT5 also specifically co-immunoprecipitates with CCR4 and CAF1 (Figure 4B; data not shown), suggesting that it too is part of the CCR4–NOT complex.

We also showed that NOT1, CCR4 and CAF1 co-purified through three different chromatographic steps using a CAF1–6His fusion to aid in the isolation of the CCR4 complex. This purified CCR4 complex containing the CAF1–6His fusion was eluted from a Superose 6 column with an estimated mol. wt of 1.2×10^6 Da. During the purification, the majority of CCR4 was found to be associated with CAF1–6His. Determination of the size of the CCR4 complex by gel filtration from a wild-type strain indicated that CCR4 and CAF1 were part of large complexes with estimated mol. wts of 1.9×10^6 and 1.0×10^6 Da. We have not been able to isolate the 1.9×10^6 Da CCR4 complex using the 6His-tagged CAF1 or CCR4, partly as the result of reduced levels of the 1.9×10^6 Da complex in these strains (unpublished observations). It is

also possible that we would not be able to isolate the larger CCR4 complex using the methodology employed in this report.

These findings confirm that the CCR4 complex is truly a multi-subunit complex. The immunoprecipitation results, the co-purification of NOT1 with the CCR4 complex, the two-hybrid analysis and the previous studies on the NOT complex strongly implicate the NOT2, 3 and 4 proteins as being components of the 1.2×10^6 Da CCR4 complex, which can be considered the core CCR4–NOT complex. It remains possible, however, that other forms of the NOT complex may exist, especially since a previously identified NOT complex was found to be only 6×10^5 Da in size (Collart and Struhl, 1994).

By several criteria, the CCR4 complexes appear distinct from the yeast holoenzyme. First, neither CCR4 nor CAF1 were found to be in purified preparations of the yeast holoenzyme (Draper *et al.*, 1995). Second, SRB5 migrated in a complex that was slightly smaller than the 1.9×10^6 Da CCR4 complex (Figure 5B). Third, SRB proteins did not co-purify with the 1.2×10^6 Da complex. A number of other proteins were checked for their presence in the 1.2×10^6 Da complex or for their ability to co-immunoprecipitate with CCR4 or CAF1. RPB1, MOT1, SPT6, SPT10, ADA2, SIN3, SIN4 and several SNF/SWI proteins were all shown not to be part of the CCR4 complex (Denis *et al.*, 1994, unpublished observations). These results place the CCR4 complex, containing the NOT proteins, as a unique and novel transcriptional regulatory group of proteins.

The role of the CAF1 protein in this complex was elucidated partly through the analysis of the effects of *caf1* defects on the association of CCR4 protein with the complex. Disruption of *caf1* effectively removed CCR4 protein from the 1.9×10^6 and 1.2×10^6 Da complexes. Correspondingly, CCR4 did not immunoprecipitate well with the NOT proteins in a *caf1* background. In contrast, a *ccr4* disruption did not affect CAF1 immunoprecipitation with the NOT proteins (Figure 3B) nor did it affect CAF1 association in the 1.9×10^6 Da complex (unpublished observation). These data indicate that CCR4 association in the complex depends on the presence of CAF1. Consistent with this conclusion is the observation that high copy expression of *CCR4* can complement a *caf1* defect (Hata *et al.*, 1997); increased levels of CCR4 would be able to associate by mass action in the CCR4 complex even in the absence of CAF1. High copy expression of *CAF1* cannot complement a *ccr4* defect (Hata *et al.*, 1997), however, apparently because CCR4 plays an essential role that increased levels of CAF1 cannot duplicate.

The NOT complex is functionally associated with the CCR4 complex

The previous studies on the *NOT* genes clearly demonstrated that they played a negative regulatory role in gene transcription. Our finding that this complex physically associates with the CCR4 complex would suggest that it should also be positively involved in gene transcription. By examining the defects of the *NOT* genes on *ADH2* expression, we were able to demonstrate that mutations in the *NOT* genes, with the exception of *NOT3*, caused a reduction of *ADH2* gene expression under glucose-derepressed conditions. This result not only establishes

functional similarity between the NOT proteins and those in the CCR4 complex but also suggests that the NOT complex is involved in activation of gene transcription.

A positive role for the NOT complex in gene transcription was demonstrated further by the observation that a *not4* disruption suppressed the ability of an *spt10* mutation to cause enhanced *ADH2* expression under glucose growth conditions. The only other known alleles which confer this phenotype are *ccr4*, *caf1* and *dbf2*, all components of the CCR4 complex (Liu *et al.*, 1997). Moreover, the expression of the *CYC1–lacZ* reporter gene, containing the upstream binding site for the HAP2, 3, 4 and 5 proteins, was reduced by defects in the *NOT* genes. In this case, the defects in the *NOT* genes reduced *CYC1–lacZ* expression as did defects in *CCR4* and *CAF1*. This reduction of *CYC1–lacZ* expression by the *not* alleles occurred primarily under non-fermentative growth conditions. Like CCR4 and CAF1, the NOT proteins may play a special role in aiding the expression of non-fermentative genes.

In addition to their similar effects on non-fermentative gene expression, the CCR4 complex components and the NOT proteins shared other phenotypic similarities. Mutations in all of these genes except that of *NOT3* resulted in increased caffeine sensitivity. This phenotype appears to be the result of impaired formation of the cell wall (Levin and Bartlett-Heubusch, 1992). Moreover, the cold-sensitive phenotype of *ccr4* and the ts phenotypes of *dbf2*, *caf1*, *not2* and *not4* were all suppressed by osmotic stabilizing agents such as sorbitol, confirming a defect caused by the alleles in terms of cell wall integrity. *ccr4*, *caf1*, *dbf2*, *not2*, *not3* and *not4* alleles were also sensitive to staurosporine, indicative of a cell wall integrity problem. Increased sensitivity to mono- and divalent cations is also a phenotype associated with *ccr4*, *caf1* and *dbf2* alleles, and a similar sensitivity was observed for the *not* alleles.

Whereas the CCR4 complex previously had been ascribed a positive role in gene expression, its association with the NOT proteins implicates them in affecting gene expression in a negative way as well. Previous data have indicated that *ccr4* mutations can negatively affect gene expression in the methionine biosynthetic pathway (McKenzie *et al.*, 1993). In this case, a *ccr4* mutation acted in a manner similar to such other negative regulators as SPT21, RPD3 and RPD1 (SIN3). Also, *ccr4* and *caf1* alleles cause increased resistance to X-ray radiation, presumably by releasing negative control of genes involved in the *RAD51* and *RAD52* pathway (Schild, 1995). Furthermore, the original identification of a *caf1* mutation (*pop2*) involved its negative control of *PGK1* expression during stationary phase (Sakai *et al.*, 1992). We further showed that *CCR4*, *CAF1* and *DBF2* negatively affect *FUS1–lacZ* expression in the same manner as did the *NOT* genes (Cade and Errede, 1994; Collart and Struhl, 1994). These observations indicate that the CCR4 complex components, like the NOT proteins, can play negative roles in controlling gene expression. Therefore, the protein association of the NOT proteins with components of the CCR4 complex results in overall similar control of gene expression and other processes.

Notwithstanding the above-described similarities, the *CCR4*, *CAF1*, *DBF2* and *NOT* genes were found to differ in their effects in some cases. This was observed most

Table III. Yeast strains

Strains	Relevant genotypes
808-5c	*MATα spt10::LEU2 ura3 his3 leu2 trp1*
KY803	*MATa trp1Δ1 ura3-52 gcn4 leu2::PET56*
KY803-1-1	isogenic to KY803 except *ccr4::URA3*
KY803-c1-1	isogenic to KY803 except *caf1::LEU2*
MY8	isogenic to KY803 except *not1-2* and *MATα*
MY16	isogenic to KY803 except *not2-1*
MY25	isogenic to KY803 except *not3-2*
MY508	isogenic to KY803 except *not3::URA3*
MY537	isogenic to KY803 except *not4::URA3*
KY803-Δ3	isogenic to KY803 except *adh1::URA3*
MY8-Δ1	isogenic to MY8 except *adh1::URA3*
MY16-Δ1	isogenic to MY16 except *adh1::URA3*
MY25-Δ1	isogenic to MY25 except *adh1::URA3*
612-1d-n4	*MATa adh1-11 ura3 his3 trp1 leu2 not4::URA3*
EGY188	*MATa adh1-11 ura3 his3 trp1 LexA–LEU2*
EGY188-c1	isogenic to EGY188 except *caf1::URA3*
EGY188-1	isogenic to EGY188 except *ccr4::URA3*
EGY188-d2	isogenic to EGY188 except *dbf2::LEU2*

obviously with the *HO–lacZ* and *FKS1–lacZ* reporter genes and with *HIS3* expression. These data suggest that although all the CCR4 complex components can share common functions, the individual components of this complex can behave differently in regulating different genes.

The fact that these various proteins can affect expression both positively and negatively suggests that the role of CCR4, CAF1 and the associated NOT proteins may be more versatile than previously indicated. The demonstration that CCR4 acts at the *ADH2* locus at a post-chromatin remodeling step (Verdone *et al.*, 1997) is consistent with the model that the NOT proteins act to regulate TATA box-binding protein (TBP) use of non-consensus TATAA sequences (Collart and Struhl, 1994; Collart, 1996). The function of the CCR4–NOT complex is also clearly affected by the sequences that lie upstream of the TATAA (Figure 7). It is likely that sequence-specific activator binding or chromatin structure influences the NOT proteins and other CCR4 complex components in their mode of action. Because of the size of the CCR4 complex and the number of its components, it is highly likely that individual factors, while showing overall functional similarity to other components in the complex, will play somewhat different roles in transcription. Individual proteins may be the targets of different regulatory factors and regulatory processes. For instance, DBF2 is a cell cycle-regulated protein kinase, and defects in *DBF2* cause a telophase block. *CAF1* and *CCR4* are themselves not cell cycle regulated, and mutations in them cause only a partial late mitotic defect (Liu *et al.*, 1997). Clearly, the CCR4 transcriptional complex does not act by itself, and identifying its contacts with the several other known protein complexes involved in transcription remains a major focus to understanding how the CCR4 complex and its individual proteins function.

Materials and methods

Strains and culture
Yeast strains are listed in Table III. Growth on YD solid medium was done with Petri plates containing YEP (1% yeast extract and 2%

bactopeptone) supplemented with 2% glucose and 2% bactoagar. β-Galactosidase assays were conducted as described (Cook *et al.*, 1994) on minimal medium lacking uracil that was supplemented either with 8% glucose or with 2% each of glycerol and ethanol. ADH II assays were conducted as described (Cook *et al.*, 1994).

Immunoprecipitation

Immunoprecipitations were carried out as described previously (Draper *et al.*, 1994; Liu *et al.*, 1997). To isolate the CCR4 complex for protein sequencing, yeast whole cell extracts prepared from a 400 ml overnight culture were mixed with 20 µl of affinity-purified LexA antibody for 45 min. To this was added 300 µl of a 50% protein A–agarose slur, and the incubation was continued for an additional 30 min. The resulting immunoprecipitate was resuspended in 150 µl of 2× SDS sample buffer and boiled for 5 min. The sample was divided and loaded onto three lanes. After electrophoresis, the gel was stained in Coomassie blue solution for 2 h and destained overnight. The protein bands of interest were excised and subjected to mass spectrometric analysis. Immunoblot analysis was carried out according to the described procedures (Liu *et al.*, 1997). The immunoblot results were analyzed by an Arcus II Scanner (Agfa-Gevaert, N.V., UK) and Adobe Photoshop 3.0 (Adobe Systems Inc., USA).

Protein identification by mass spectrometry

Techniques and strategy of analysis were as previously described (Shevchenko *et al.*, 1996). Gel pieces were washed, 'in-gel' reduced, S-alkylated, and protein enzymatically degraded with trypsin as described (Wilm *et al.*, 1996). After 3 h, ~2% of the digest product was applied on a micro-crystalline layer—a mixture of α-cyano-4-hydroxycinnamic acid and nitrocellulose (Jensen *et al.*, 1996)—and analyzed by MALDI-time of flight mass spectrometry. The mass spectrometer (Bruker Reflex, Bruker-Franzen, Bermen, Germany) was equipped with delayed ion extraction. For peptide sequencing by nanoelectrospray mass spectrometry, the remaining product was extracted, concentrated and desalted on a 100 nl R2 Poros microcolumn, and eluted in 2×0.5 µl of 60% methanol, 5% formic acid into a nanoelectrospray spraying needle as described (Wilm and Mann, 1996; Wilm *et al.*, 1996). Analyses were performed on a triple quadrupole mass spectrometer (API III, Perkin-Elmer Sciex, Toronto, Canada). Database searches by peptide mass maps and by peptide sequence tags (Mann and Wilm, 1994) were performed with the program PeptideSearch using a comprehensive non-redundant database currently containing >230 000 entries.

Purification of the CCR4 complex

Yeast whole cell extracts were prepared by a modification of the method of Liu *et al.* (1997) in which 3× buffer A [50 mM Tris-OAc, pH 7.9/150 mM KOAc/20% glycerol/0.2% Tween-20/2 mM β-mercaptoethanol (β-ME)/2 mM MgOAc plus protease inhibitors] was used to resuspend cell pellets (140 g of wet cells). The clear extract (120 ml) was applied to a 4 ml Ni^{2+}-NTA column. The bound proteins were eluted in 250 mM imidazole in buffer A. The resulting Ni^{2+} eluate (12 ml) was applied to a Mono Q HR5/5 column and the protein fractions were then eluted in a 20 ml linear 100–2000 mM gradient of KOAc in buffer B: 50 mM Tris-OAc, pH 7.9/100 mM KOAc/20% glycerol/0.02% Tween-20/1 mM β-ME/2 mM MgOAc/1 mM EDTA, plus protease inhibitors including 2 µl/ml of leupeptin (2 mg/ml), pepstatin A (1 mg/ml), chymostatin (5 mg/ml) and benzamidine (500 mg), and 10 µl/ml of 500 mM phenylmethylsulfonyl fluoride (PMSF). The fractions containing CCR4 and CAF1–6His were pooled (2 ml in total) and subjected to ultrafiltration using a Centricon 10 device (Amicon, MA). The concentrated protein sample (200 µl) was applied to a Superose 6 HR10/30 column equilibrated in buffer G (50 mM Tris-OAc, pH 7.9/150 mM KOAc/10% glycerol/0.02% Tween-20/1 mM dithiothreitol/1 mM EDTA/2 mM MgOAc) plus protease inhibitors. The protein fractions were collected as 0.5 ml/fraction and stored at –80°C while part of the materials (10 µl for Ni^{2+}-NTA and Mono Q eluate, and 25 µl for Superose 6 fractions) was subjected to immunoblot analysis using CCR4, CAF1 and NOT1 antibodies.

Gel filtration chromatography

The Superose 6 columns HR10/30 and HR16/50 were packed with Superose 6 media, prep grade, according to the manufacturer's instructions (Pharmacia). A molecular weight standard mixture was used to calibrate the Superose 6 columns. The calibration for the HR10/30 column in buffer G plus protease inhibitors was: exclusion volumn (blue dextran, 2000 kDa) at 10 ml; thyroglobulin (669 kDa) at 15 ml; bovine serum albumin (BSA) (66 kDa) at 17.5 ml. For the HR16/50 column,

the calibration was: blue dextran at 38.6 ml; thyroglobulin at 59 ml; amylase (200 kDa) at 65 ml; BSA at 69 ml; carbonic anhydrase (29 kDa) at 72.7 ml.

To analyze the CCR4 complex using gel filtration chromatography, yeast whole cell extracts prepared from a 500 ml overnight glucose-grown culture in 3× buffer A were first clarified by ultracentrifugation in a SW65 rotor at 45 000 r.p.m. for 60 min. Then 200 µl of the clear extracts were loaded onto a Superose 6 column. The resulting 1 ml fractions were subjected to Western blot analysis. For the DNase treatment experiments, yeast whole extracts were prepared in EDTA-free 3× buffer A. After ultracentrifugation, 7.5 µl of DNase I (61.5 µg/ml) were added to 1 ml of the clear extracts and the mixture was incubated at room temperature for 7 min. Then 200 µl of the mixture was analyzed on the Superose 6 column and 30 µl of the 1 ml fractions were subjected to immunoblot analysis.

References

Breeden,L. and Nasmyth,K. (1987) Cell cycle control of the yeast *HO* gene: *cis*- and *trans*-acting regulators. *Cell*, **48**, 389–397.

Cade,R.M. and Errede,B. (1994) *MOT2* encodes a negative regulator of gene expression that affects basal expression of pheromone-responsive genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **14**, 3139–3149.

Collart,M.A. (1996) The *NOT*, *SPT3*, and *MOT1* genes functionally interact to regulate transcription at core promoters. *Mol. Cell. Biol.*, **16**, 6668–6676.

Collart,M.A. and Struhl,K. (1993) CDC39, an essential nuclear protein that negatively regulates transcription and differentially affects the constitutive and inducible HIS3 promoters. *EMBO J.*, **12**, 177–186.

Collart,M.A. and Struhl,K. (1994) *NOT1* (*CDC39*), *NOT2* (*CDC36*), *NOT3*, and *NOT4* encode a global-negative regulator of transcription that differentially affects TATA-element utilization. *Genes Dev.*, **8**, 525–537.

Cook,W.J., Chase,D., Audino,D.C. and Denis,C.L. (1994) Dissection of the ADR1 protein reveals multiple, functionally redundant activation domains interspersed with inhibitory regions: evidence for a repressor binding to the ADR1^c region. *Mol. Cell. Biol.*, **14**, 629–640.

Denis,C.L. (1984) Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics*, **108**, 833–844.

Denis,C.L. and Malvar,T. (1990) The *CCR4* gene from *Saccharomyces cerevisiae* is required for both nonfermentative and spt-mediated gene expression. *Genetics*, **124**, 283–291.

Denis,C.L., Draper,M.P., Liu,H.-Y., Malvar,T., Vallari,R.C. and Cook,W.J. (1994) The yeast CCR4 protein is neither regulated by nor associated with the SPT6 and SPT10 proteins and forms a functionally distinct complex from that of the SNF/SWI transcription factors. *Genetics*, **138**, 1005–1013.

Draper,M.P., Liu,H., Nelsbach,A.H., Mosley,S.P. and Denis,C.L. (1994) CCR4 is a glucose-regulated transcription factor whose leucine-rich repeat binds several proteins important for placing CCR4 in its proper promoter context. *Mol. Cell. Biol.*, **14**, 4522–4531.

Draper,M.P., Salvadore,C. and Denis,C.L. (1995) Identification of a mouse protein whose homolog in *Saccharomyces cerevisiae* is a component of the CCR4 transcriptional regulatory complex. *Mol. Cell. Biol.*, **15**, 487–495.

Grant,P.A. *et al.* (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev.*, **11**, 1640–1650.

Guarente,L. and Mason,T. (1983) Heme regulates transcription of the *CYC1* gene of *S.cerevisiae* via an upstream activation site. *Cell*, **32**, 1279–1286.

Hata,H., Mitsui,H., Liu,H., Bai,Y., Denis,C.L., Shimizu,Y. and Sakai,A. (1997) Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*. *Genetics*, in press.

Igual,J.C., Johnson,A.L. and Johnston,L.H. (1996) Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J.*, **15**, 5001–5013.

Irie,K., Yamaguchi,K., Kawase,K. and Matsumoto,K. (1994) The yeast *MOT2* gene encodes a putative zinc finger protein that serves as a global negative regulator affecting expression of several categories of genes, including mating-pheromone-responsive genes. *Mol. Cell. Biol.*, **14**, 3150–3157.

Jensen,O.N., Podtelejnikov,A. and Mann,M. (1996) Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun. Mass Spectrom.*, **10**, 1371–1378.

Lamond,A.I. and Mann,M. (1997) Cell Biology and Genome Projects— a concerted strategy for charaterizing multi-protein complexes using mass spectrometry. *Trends Cell Biol.*, **7**, 139–142.

Levin,D.E. and Bartlett-Heubusch,E. (1992) Mutants in the *S.cerevisiae PKC1* gene display a cell cycle-specific osmotic stability defect. *J. Cell Biol.*, **116**, 1221–1229.

Liu,H., Toyn,J.H., Chiang,Y.-C., Draper,M.P., Johnston,L.H. and Denis,C.L. (1997) DBF2, a cell cycle-regulated protein kinase, is physically and functionally associated with the CCR4 transcriptional regulatory complex. *EMBO J.*, **16**, 5289–5298.

Mann,M. and Wilm,M. (1994) Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal. Chem.*, **66**, 4390–4399.

McKenzie,E.A., Kent,N.A., Dowell,S.J., Moreno,F., Bird,L.E. and Mellor,J. (1993) The centromere and promoter factor, 1, CPF1, of *Saccharomyces cerevisiae* modulates gene activity through a family of factors including SPT21, RPD1 (SIN3), RPD3 and CCR4. *Mol. Gen. Genet.*, **240**, 374–386.

Natsoulis,G., Dollard,C., Winston,F. and Boeke,J.D. (1991) The products of the *SPT10* and *SPT21* genes of *Saccharomyces cerevisiae* increase the amplitude of transcriptional regulation at a large number of unlinked loci. *New Biol.*, **3**, 1249–1259.

Neubauer,G., Gottschalk,A., Fabrizio,P., Seraphin,B., Luhrmann,R. and Mann,M. (1997) Identification of the proteins of the yeast U1 small nuclear ribonucleoprotein complex by mass spectrometry. *Proc. Natl Acad. Sci. USA*, **94**, 385–390.

Oberholzer,U. and Collart,M.A. (1998) Characterization of *NOT5* that encodes a new component of the NOT protein complex. *Gene*, in press.

Sakai,A., Chibazakura,T., Shimizu,Y. and Hishinuma,F. (1992) Molecular analysis of *POP2* gene, a gene required for glucose-derepression of gene expression in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **20**, 6227–6233.

Schild,D. (1995) Suppression of a new allele of the yeast *RAD52* gene by overexpression of *RAD51*, mutations in *srs2* and *ccr4*, or mating-type heterozygosity. *Genetics*, **140**, 115–127.

Shevchenko,A. *et al.* (1996) Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl Acad. Sci. USA*, **93**, 14440–14445.

Toyn,J.H., Araki,H., Sugino,A. and Johnston,L.H. (1991) The cell-cycle-regulated budding yeast gene *DBF2*, encoding a putative protein kinase, has a homologue that is not under cell-cycle control. *Genetics*, **104**, 63–70.

Verdone,L., Cesari,F., Denis,C.L., Di Mauro,E. and Caserta,M. (1997) Factors affecting *S.cerevisiae ADH2* chromatin remodeling and transcription. *J. Biol. Chem.*, **272**, 30828–30834.

Wade,P.A., Werel,W., Fentzke,R.C., Thompson,N.E., Leykam,J.F., Burgess,R.R., Jaehning,J.A. and Burton,Z.F. (1996) A novel collection of accessory factors associated with yeast RNA polymerase II. *Protein Expr. Purif.*, **8**, 85–90.

Wilm,M. and Mann,M. (1996) Analytical properties of the nanoelectrospray ion source. *Anal. Chem.*, **68**, 1–8.

Wilm,M., Shevchenko,A., Houthaeve,T., Breit,S., Schweigerer,L., Fotsis,T. and Mann,M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature*, **379**, 466–469.

Wilson,C.J., Chao,D.M., Imbalzano,A.N., Schnitzler,G.R., Kingston,R.E. and Young,R.A. (1996) RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell*, **84**, 235–244.