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

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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 1.2×106 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 Mg2+ (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
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 nanoelectrospray (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-immunoprecipitated with LexA–NOT1 (Figure 3A, lane 3) while NOT1 was 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 immunoprecipitate the LexA–NOT2 fusion while antibodies raised against either CCR4 or CAF1 were used to bring down CCR4 and CAF1, respectively. The resulting immunoprecipitates were subjected to SDS–PAGE, followed by Western blot analysis (Figure 3A and B). Immunoprecipitating 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-immunoprecipitate 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
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-immunoprecipitate 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 antibody (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 immunoprecipitated 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 interacted 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...
CCR4–NOT complex

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 caf1Δ-deleted strain (lanes 1, 3 and 5) or a ccr4Δ-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 1.0×10^6 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),

Table I. Two-hybrid interaction assay

<table>
<thead>
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<th>β-Gal activity (U/mg)</th>
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<tr>
<td>B42–NOT1</td>
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<tr>
<td>LexA–NOT1</td>
</tr>
<tr>
<td>LexA–NOT2</td>
</tr>
<tr>
<td>LexA–CCR4</td>
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<tr>
<td>LexA–CAF1</td>
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<td>LexA</td>
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LexA–CCR4, –NOT1 and –NOT2 contain full-length CCR4, NOT1 and NOT2. LexA–CAF1 contains residues 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.
nor on the SRB5 protein to migrate at $1.7 \times 10^6$ 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 $6 \times$His was integrated into the genome at the TRP1 locus. This CAF1–6His gene was able to complement the defect of caf1Δ (Liu et al., 1997). The extracts prepared from this strain were first put onto a Ni$^{2+}$-NTA column, and the bound proteins were 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 $1.2 \times 10^6$ Da following the Superose 6 gel filtration chromatography (Figure 6), corresponding closely to the $1.0 \times 10^6$ 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 $1.9 \times 10^6$ and $1.2 \times 10^6$ 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

<table>
<thead>
<tr>
<th>Strains</th>
<th>ADH II</th>
<th>spt10</th>
<th>Caffeine 8 mM</th>
<th>37°C YD</th>
<th>37°C YD 1 M sorbitol</th>
<th>Mg(^2+) 750 mM</th>
<th>Stauro 1 mg/ml</th>
<th>3 AT 20 mM</th>
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<tbody>
<tr>
<td>wt</td>
<td>2400</td>
<td>91</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ccr4</td>
<td>400</td>
<td>23</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>caf1</td>
<td>1000</td>
<td>7</td>
<td>w</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>w/–</td>
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<tr>
<td>not1</td>
<td>1300</td>
<td>78</td>
<td>–</td>
<td>w</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>not2</td>
<td>340</td>
<td>86</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>not3</td>
<td>2500</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
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<tr>
<td>not4</td>
<td>1200</td>
<td>13</td>
<td>w</td>
<td>–</td>
<td>w</td>
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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-amino-triazole (3AT) using strains isogenic to KY803 (wt) containing the YC88-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-AΔ; not1, MY8-AΔ; not2, MY16-AΔ; not3, MY25-AΔ; not4, 612-1d-AΔ; and for spt10 ADH II assays the strains were: wt, spt10 segregants from cross 808-5c and 612-1d-s4; not1, spt10 not1-2 segregants from cross MY8/AΔ and 1366-4a; not2, spt10 not2 segregants from cross 808-5c and MY16-AΔ; 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-galactosidase 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 were 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. RBP1, 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-repressed 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 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%
bacopetopnone) 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 according to described procedures (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 slurry, 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 and Mann, 1996; Wilm et al., 1997). After 3 h, 2% of the digest protein was applied onto a microcrystalline trypsin layer in 450 μl of 0.1% formic acid and 5% 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 7.9/100 mM KOAc/20% glycerol/0.2% Tween-20/0.2% 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 on a 100 ml R2 Poros microcolumn, and eluted in 200 μl of 0.5 M 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.

**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 volume (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 7.2 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.

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H.-Y.Liu et al.


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