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Identification of a Mouse Protein Whose Homolog in *Saccharomyces cerevisiae* Is a Component of the CCR4 Transcriptional Regulatory Complex†

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The CCR4 protein from *Saccharomyces cerevisiae* is a component of a multisubunit complex that is required for the regulation of a number of genes in yeast cells. We report here the identification of a mouse protein (mCAF1 [mouse CCR4-associated factor 1]) which is capable of interacting with and binding to the yeast CCR4 protein. The mCAF1 protein was shown to have significant similarity to proteins from humans, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *S. cerevisiae*. The yeast gene (y*CAF1*) had been previously cloned as the *POP2* gene, which is required for expression of several genes. Both yCAF1 (POP2) and the *C. elegans* homolog of CAF1 were shown to genetically interact with CCR4 in vivo, and yCAF1 (POP2) physically associated with CCR4. Disruption of the *CAF1* (*POP2*) gene in yeast cells gave phenotypes and defects in transcription similar to those observed with disruptions of *CCR4*, including the ability to suppress *spt10*-enhanced *ADH2* expression. In addition, yCAF1 (POP2) when fused to LexA was capable of activating transcription. mCAF1 could also activate transcription when fused to LexA and could functionally substitute for yCAF1 in allowing *ADH2* expression in an *spt10* mutant background. These data imply that CAF1 is a component of the CCR4 protein complex and that this complex has retained evolutionarily conserved functions important to eukaryotic transcription.

The general transcription factor CCR4 from *Saccharomyces cerevisiae* is required for transcription of a number of genes, including the yeast *ADH2* gene (encoding the glucose-repressible alcohol dehydrogenase II protein [ADH II]) (7, 10, 20, 27). Strains containing deletions of *CCR4* fail to grow at elevated temperatures on a nonfermentative medium and also display a growth defect at 16°C on a glucose-containing medium (10, 12). CCR4 is required for the elevated expression at the *ADH2* locus and for the altered transcriptional initiation at the *his4-912*Δ locus that results from defects in the *SPT6* and *SPT10* genes (10). The *SPT6* and *SPT10* genes encode factors that are responsible for maintaining proper transcriptional control over a wide range of genes and have been implicated in the maintenance of chromatin structure (7, 11, 21, 29). *spt6* mutations also suppress defects in the SNF/SWI family of factors that appear to aid activator accessibility to nucleosomal DNA (6, 13, 33). CCR4, however, is neither complexed with nor regulated by the SPT6 and SPT10 proteins (9). Moreover, the CCR4 protein complex is distinct from that of the SNF/SWI factors. CCR4 also appears to act in a manner mechanistically different from that of the SNF/SWI proteins (9).

CCR4 is an 837-amino-acid protein (19). When fused to LexA, CCR4 can function as a weak activator of transcription (12). Two N-terminal regions of CCR4 are responsible for this activation function (12). In the middle of the CCR4 protein lie five leucine-rich repeats (LRRs), which are protein binding domains (2, 17) required for CCR4 function in vivo (19). CCR4 associates with at least four other proteins, and the LRR specifically binds two of these, 185 and 195 kDa in size (12). The 185- and 195-kDa species may be modified forms of the same protein.

The pleiotropic nature of defects in CCR4 and the evolutionary conservation of many of the core components of the RNA polymerase II transcription apparatus (24, 32) led us to postulate that CCR4 and its associated factors might be found in other eukaryotes. The strong interaction between CCR4 and the 185- and 195-kDa proteins suggested also that these specific protein contacts might be evolutionarily conserved. We have tested this hypothesis by seeking to identify proteins from mice that interacted with CCR4. We present evidence here that the mCAF1 (mouse CCR4-associated factor 1) protein interacts and binds to the yeast CCR4-containing complex. The mCAF1 protein shares a high degree of sequence similarity with proteins found in *S. cerevisiae*, humans, *Caenorhabditis elegans*, and *Arabidopsis thaliana*. The yeast protein (yCAF1) is a CCR4-associated factor, and mCAF1 retains a number of functions of the yCAF1 protein. These data suggest that the CCR4 complex plays an important and conserved role in eukaryotic transcription.

MATERIALS AND METHODS

Yeast and *Escherichia coli* strains, growth conditions, and enzyme assays. Yeast strains are listed in Table 1. Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium (2% Bacto Peptone, 1% yeast extract, and 20 mg each of adenine and uracil per liter, containing either 8% glucose or 2% ethanol as a carbon source) have been described elsewhere (5). YD solid medium contained YEP supplemented to 2% glucose and 2.5% agar. β-Galactosidase assays were conducted on yeast extracts as described previously (5). ADH II activity was assayed as described previously (8). Because yeast cells expressing LexA-CCR4 or GAL4 transactivation domain fusions of CCR4, yCAF1, or mCAF1 were observed to undergo loss of activity with prolonged maintenance on selective plates, assays were conducted within as short a time as possible on new transformants or freshly streaked-out colonies. Values represent the averages of at least three separate determinations.

Plasmids. LexA202 and LexA87 plasmids are 2μm-based plasmids and have been previously described (3, 24). The 34 reporter plasmid is a 2μm-based plasmid containing eight LexA operator sites controlling the *lacZ* gene (3, 5). The GAL4 transactivation domain vector (pPC86), as well as the murine cDNA library in pPC86, have been described elsewhere (4). The vector pJG4-5 directs

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TABLE 1. Yeast strains used in this study

Strain	Genotype
EGY188	*MAT***a** *his3 leu2 trp1 ura3 lexAop-LEU2*
EGY191	*MAT*α *his3 leu2 trp1 ura3 lexAop-LEU2*
EGY191-2	Isogenic to EGY191 except *caf1*::*LEU2*
MD9-7c	*MAT*α *adh1-11 his3 trp1 ura3 ccr4-10*
MD9-7c+	Isogenic to MD9-7c except *TRP1*::*CCR4*
147-6d	*MAT*α *adh1-11 his4 leu2 trp1 ura1 spt6-1*
992-6a	*MAT***a** *adh1-11 his3 trp1 ura3 caf1*::*LEU2*
994-2	*MAT*α *adh1-11 his3 leu2 ura3 spt10*::*TRP1*
1005-2-3c	*MAT***a** *adh1-11 his3 ura3 trp1 leu2 caf1*::*LEU2 spt10*::*TRP1*
935-1-6	Isogenic to 935-1 except *caf1*::*LEU2*
935-1	*MAT***a** *adh1-11 his3 ura3 trp1 leu2 ccr4-10*
935-2	*MAT*α *adh1-11 his3 leu2 ura3*
935-2-3	Isogenic to 935-2 except *caf1*::*LEU2*

the synthesis of proteins that carry at their amino termini the influenza virus HA1 epitope tag, the B42 acidic activation domain, and the simian virus 40 nuclear localization signal (35). LexA-B42 contains an *E. coli*-derived polypeptide that activates transcription in yeast cells (24).

Plasmid constructions. The construction of all LexA-CCR4 fusions used has been described elsewhere (12), as have the LexA-ADR1 fusions (5). The GAL4-yCAF1 and LexA-yCAF1 constructs were made by placing a *Bam*HI (cuts at bp −14)-*Hin*dIII (cuts 3′ to the y*CAF1* gene) fragment, in which the *Hin*dIII site was made blunt ended by using the large subunit of *E. coli* DNA polymerase (Klenow fragment) as described previously (26), into the *Bam*HI-*Sal*I site, at which the *Sal*I had been made blunt ended, of either the pPC86 or LexA(202-2) vector. The resulting pPC86 construct was then cut with *Sal*I (made blunt ended with the Klenow fragment) and *Sma*I and religated to place the coding sequence for yCAF1 in frame with GAL4. The GAL4-CCR4-1-837 fusion was made by placing an *Eco*RI-*Bgl*II fragment from pTM10 (19) containing the complete coding sequence for CCR4 into the *Eco*RI-*Bam*HI site of the pPC86 vector. The GAL4-*C. elegans* CAF1 (cCAF1) fusion was constructed by placing a blunt-ended *Eco*RV-*Hin*dIII fragment from plasmid CM21F10 containing the coding sequence for c*CAF1* into the *Eco*RI site of pPC86 made blunt ended with the Klenow fragment. The HA1-B42-mCAF1 fusion was constructed by placing a *Mlu*I-*Bam*HI fragment from the GAL4-mCAF1 clone, made blunt ended by using the Klenow fragment, into the filled-in *Eco*RI site of pJG4-5.

LexA-yCAF1-1-181 was constructed by inserting the *Bam*HI-*Eco*RI (cuts at bp +544; blunt ended with the Klenow fragment) fragment of the y*CAF1* gene into the *Bam*HI-*Sal*I (blunt ended with the Klenow fragment) sites of LexA(202-2) (5). The fusion LexA-yCAF1-1-181/mCAF1-1-285 was constructed by first cloning the *Bam*HI-*Hin*dIII fragment of y*CAF1* into pUC18 (*Eco*RI site filled in with the Klenow fragment). The *Mlu*I (blunt ended with the Klenow fragment)-*Hin*dIII fragment of m*CAF1* that contains residues 12 to 285 of mCAF1 from a GAL4-mCAF1 plasmid was then used to replace the *Eco*RI (residue 182 of yCAF1)-*Hin*dIII fragment of the yCAF1-pUC18 derivative. This pUC18 vector was then cut with *Bam*HI-*Hin*dIII, the *Hin*dIII site was filled in with the Klenow fragment, and the fragment was inserted into LexA(202-4) which had been cut with *Bam*HI and *Sal*I (filled in with the Klenow fragment). The fusion LexA-yCAF1-127-444 was constructed by cutting a pUC-based vector which contained the y*CAF1* gene with *Pst*I (bp +351)-*Hin*dIII (cuts 3′ of the gene). This fragment was inserted into pUC18 at the *Pst*I-*Hin*dIII site. The *Bam*HI-*Hin*dIII (blunt ended) fragment containing y*CAF1* was then inserted into LexA(202-2) at the *Bam*HI-*Sal*I (blunt ended with the Klenow fragment). GAL4-yCAF1-80-444 was constructed by placing the *Hin*cII (cuts at bp +236)-*Hin*dIII (filled in with the Klenow fragment) into the *Sma*I site of pPC86. LexA-yCAF1-80-444 was constructed by removing the y*CAF1* sequences from GAL4-yCAF1-80-444 and inserting them into the LexA(202-3) vector (5). The *CAF1* gene was disrupted with the *LEU2* gene by cutting *CAF1* at its *Pst*I site and inserting a *Pst*I fragment containing the *LEU2* gene. This resulted in a *CAF1* allele that expressed only the N-terminal 126 amino acids of CAF1. A *CAF1* disruption was made by cutting this plasmid with *Hin*dIII and transforming the appropriate yeast strains. Southern analysis (26) was used to verify that the *CAF1* (*POP2*) gene had been disrupted. LexA-yTFIIB was constructed by cloning an *Nde*I (cuts at bp +1; blunt ended with the Klenow fragment)-*Bgl*II (3′ end of the gene) fragment of yeast TFIIB into LexA(202-1) cut with *Eco*RI (blunt ended with the Klenow fragment) and *Bam*HI (5).

Native immunoprecipitations. Immunoprecipitations were performed as described elsewhere (12). Western blotting (immunoblotting) was performed with purified polyclonal rabbit antisera raised against whole LexA protein or peptides based on the N terminus of CCR4, the N terminus of yCAF1 (SQRQASEQ HQQQNMGPQCC), or the C terminus of yCAF1 (CCKYQGVIYGIDGDQ). The antibody against GAL4 was a gift from Jim Hopper. Western analysis and silver staining were performed as described previously (31, 34).

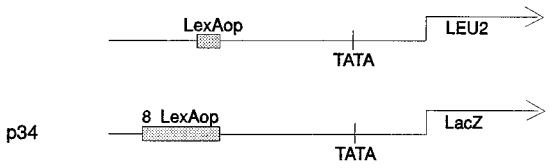

FIG. 1. Reporter genes. *LexA-LEU2* is integrated at the *LEU2* gene (35), and the LexA-LacZ plasmid contains eight LexA binding sites.

Isolation of clones, sequencing, and protein sequence analysis. The full-length y*CAF1* gene was isolated from a yeast YCp50 genomic library by colony hybridization (26), using a partial clone generously provided by M. Cusick. The full-length c*CAF1* clone was obtained from R. H. Waterson, Washington University School of Medicine. Insert DNAs containing m*CAF1* and c*CAF1* were sequenced on each strand by double-stranded sequencing with deoxynucleotides and Sequenase (U.S. Biochemical). Sequence comparison analysis was performed at the National Center for Biotechnology Information, using the BLAST network service (1). Alignments were performed by using the clustal method available in the megalign portion of the DNA STAR package (DNA STAR Inc.).

Nucleotide sequence accession numbers. The sequences of the m*CAF1* and c*CAF1* cDNAs may be obtained under GenBank/EMBL accession numbers U21855 and U21854, respectively.

RESULTS

CCR4 is capable of interacting with a mouse protein. The yeast two-hybrid system was used to isolate and characterize mouse proteins which were capable of interacting with CCR4. A yeast plasmid library constitutively expressing cDNA-encoded mouse proteins fused to the GAL4 transactivation domain (GAL4) (4) was transformed into a yeast strain containing a plasmid directing the synthesis of a LexA(1-202)-CCR4-1-837 fusion and two reporters each under the control of the LexA operator (Fig. 1). Of the transformants which exhibited activation of both reporters, only two isolates showed dependence on both the LexA-CCR4 plasmid and the GAL4-mouse cDNA fusion. Sequencing revealed that the two positive clones were identical. We named this cDNA m*CAF1*. The GAL4-mCAF1 protein did not interact with LexA alone or activate transcription by itself (Fig. 2). Also, the GAL4 activation domain did not bind to CCR4 since GAL4 or GAL4-CCR4 failed to show an interaction with LexA-CCR4 (Fig. 2). Placement of another LexA fusion, LexA-yTFIIB, with both the GAL4 transactivation domain and GAL4-mCAF1 also failed to exhibit any increase in activity over that of LexA-yTFIIB alone (Fig. 2). To further verify the basis of this interaction, we fused the *CAF1* open reading frame to the coding sequence for a different transcriptional activator, HA1-B42. B42 is an *E. coli*-derived sequence which activates transcription in yeast cells when bound to DNA (18). This HA1-B42-mCAF1 fusion construct along with LexA-CCR4 resulted in increased β-galactosidase activity over that observed with the HA1-B42-containing vector alone with LexA-CCR4 (Fig. 2).

mCAF1 is structurally similar to proteins in humans, *C. elegans*, *A. thaliana*, and *S. cerevisiae*. Sequencing of the m*CAF1* reading frame from the GAL4-mCAF1-containing plasmid and a larger cDNA that we isolated from a mouse 3T3-L1 adipocyte cDNA library revealed a complete open reading frame of 285 amino acids (Fig. 3). Also, antibody directed to the GAL4 transactivation domain specifically identified the GAL4-mCAF1 protein in yeast crude extracts at a size of 50 kDa, in good agreement with the predicted size of the GAL4-mCAF1 fusion (see Fig. 7). Database searches utilizing the amino acid sequence for mCAF1 led to the identification of proteins with a high degree of similarity from humans, *C. elegans*, *A. thaliana*, and the yeast *S. cerevisiae*. The *C. elegans* protein (cCAF1), the human protein (hCAF1), and the *A.*

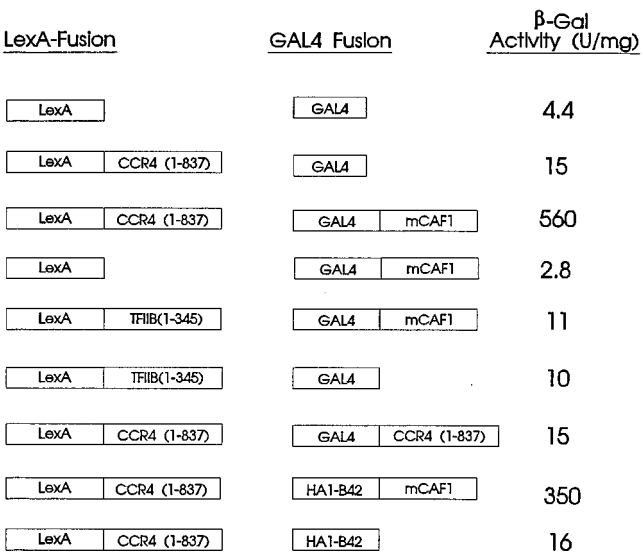

FIG. 2. Interaction of mCAF1 with CCR4. Plasmids that directed the synthesis of GAL4-tagged proteins were introduced into strain EGY188 along with the LexA fusions listed. The 34 reporter was used in all cases. Activation was monitored by β-galactosidase (β-Gal) assay in liquid cultures grown on 8% glucose except for those strains with HA1-B42-tagged proteins, which were grown in 2% galactose and 2% raffinose to induce the expression of the HA1-tagged proteins. β-Galactosidase assays were performed on at least three different transformants. All standard errors of the means were less than 20%.

thaliana protein were all found submitted in the dbest database as partial cDNA sequences. The yeast gene, y*CAF1*, was found as a complete sequence under the name *POP2. POP2* had been identified as a gene encoding a transcription factor, mutations in which augmented yeast *PGK* expression during stationary phase and reduced invertase and isocitrate lyase enzyme activity during derepression (25).

The m*CAF1* and h*CAF1* sequences displayed 99% identity at the DNA level over the 190 nucleotides of the h*CAF1* sequence available from the dbest database. Because of this near identity, the h*CAF1* sequence was not further analyzed. Instead, in order to make a broader evolutionary comparison, the c*CAF1* cDNA was obtained, sequenced in its entirety, and shown to encode a protein of 310 amino acids. Examination of the yCAF1 (POP2) protein revealed 40% identity at the amino acid level with the mCAF1 protein and 30% identity with the cCAF1 protein. The cCAF1 protein was 48% identical to mCAF1. The strongest region of identity between all three of these proteins was between residues 174 and 341 of the yeast protein, in which there was a 32% identity between the three proteins (Fig. 3). The yCAF1 (POP2) protein differed from the mCAF1 and cCAF1 proteins in that it contained an N-terminal extension of 148 residues. The *A. thaliana* CAF1 sequence was submitted to the dbest database while this report was in preparation and was not analyzed further. Besides the similarity of the CAF1 proteins to each other, they shared no similarities to other proteins in the database.

When the y*CAF1* (*POP2*) coding sequence was inserted into the GAL4 transactivation domain vector, the GAL4-yCAF1 fusion interacted in the two-hybrid system with LexA-CCR4 (Fig. 4). Also, subsequent yeast two-hybrid searches using a yeast genomic library fused to HA1-B42 (35) identified several clones that contained C-terminal segments of yCAF1 (data not shown). A significant, albeit weak, interaction between GAL4-cCAF1 and CCR4 was also observed (Fig. 4). This weak interaction may be due to the decreased abundance of cCAF1 in yeast cells, since Western analysis indicated that GAL4-cCAF1 expression was at least 10-fold less than that observed for GAL4-mCAF1 or GAL4-yCAF1 (data not shown). Why GAL4-mCAF1 interacted better with LexA-CCR4 than did GAL4-yCAF1 is not clear (see also Fig. 8).

yCAF1 and mCAF1 physically interact with CCR4. We subsequently examined whether LexA-yCAF1 could bind CCR4. When an antibody directed against LexA was used in immunoprecipitations, a band corresponding to the 70-kDa predicted size of the LexA-yCAF1 fusion was seen by Western analysis using an anti-LexA antibody (Fig. 5; compare lanes A and B). CCR4 was also coimmunoprecipitated with LexA-yCAF1, as judged by detection of a band at 97 kDa by Western analysis using an anti-CCR4 antibody (Fig. 5, lane B). We detected no CCR4 in a strain in which just LexA alone had been immunoprecipitated (Fig. 5, lane A). Further, when the CCR4 antibody was used to conduct the immunoprecipitation, LexA-yCAF1 was specifically immunoprecipitated (Fig. 6, lane C). No protein band corresponding to LexA-yCAF1 was observed in CCR4 immunoprecipitations from a strain containing just LexA (Fig. 5, lane D).

While the results presented above were indicative of a physical association between yCAF1 (POP2) and CCR4, we also wished to establish that the yCAF1 (POP2) protein at its normal physiological concentration was bound to CCR4. Immunoprecipitation of CCR4 with an antibody against CCR4 and subsequent probing of the resulting blots with the yCAF1 antibody raised against an N-terminal peptide of yCAF1 revealed the 50-kDa yCAF1 (POP2) protein running just under the immunoglobulin G (Fig. 6, lane A). The yCAF1 (POP2) protein was also identified with a yCAF1 antibody raised against a C-terminal peptide of yCAF1 (Fig. 6, lane B). Control immunoprecipitation experiments indicated that the 50-kDa protein was not detected with an anti-CAF1 antibody in extracts lacking yCAF1 (POP2) (Fig. 6, lanes D and E), that other antibodies directed against CCR4 and SPT10 did not recognize the 50-kDa yCAF1 (POP2) protein, and that other CAF1 fusion proteins were recognized specifically by the yCAF1 antibodies (data not shown). In the reverse experiment, CCR4 was found to coimmunoprecipitate with yCAF1 (POP2) when an antibody against the N terminus of yCAF1 was used for the immunoprecipitation (Fig. 6, lane C). This coimmunoprecipitation of CCR4 with yCAF1 (POP2) could also be blocked by the addition of excess yCAF1 peptide to which the yCAF1 antibody had been raised (data not shown).

We also examined whether there was a comparable physical interaction between CCR4 and mCAF1. When an antibody directed against LexA was used to immunoprecipitate LexA-CCR4 from a strain expressing the GAL4-mCAF1 fusion, the 50-kDa GAL4-mCAF1 protein was specifically detected by Western analysis using the GAL4 antibody (Fig. 7, lane A). A similar result was obtained for a strain carrying a LexA(1-87)-CCR4 fusion and GAL4-mCAF1 (data not shown). The 50-kDa GAL4-mCAF1 protein was absent when the immunoprecipitation was conducted with extracts containing only LexA-CCR4 (Fig. 7, lane D) or extracts of a strain containing LexA alone and GAL4-mCAF1 (Fig. 7, lane B). Moreover, immunoprecipitation of LexA-CCR4 from a strain containing the GAL4-CCR4 fusion failed to show an interaction in the coimmunoprecipitation experiment, indicating that the interaction was not between the GAL4 transactivation domain and LexA-CCR4 (Fig. 7, lane E). In confirmation of these results, immunoprecipitation of wild-type CCR4 from a strain containing the GAL4-mCAF1 fusion also brought down the 50-kDa mCAF1 fusion species (Fig. 7, lane F). The 50-kDa mCAF1 protein was not detected when a strain without the GAL4-mCAF1 fusion

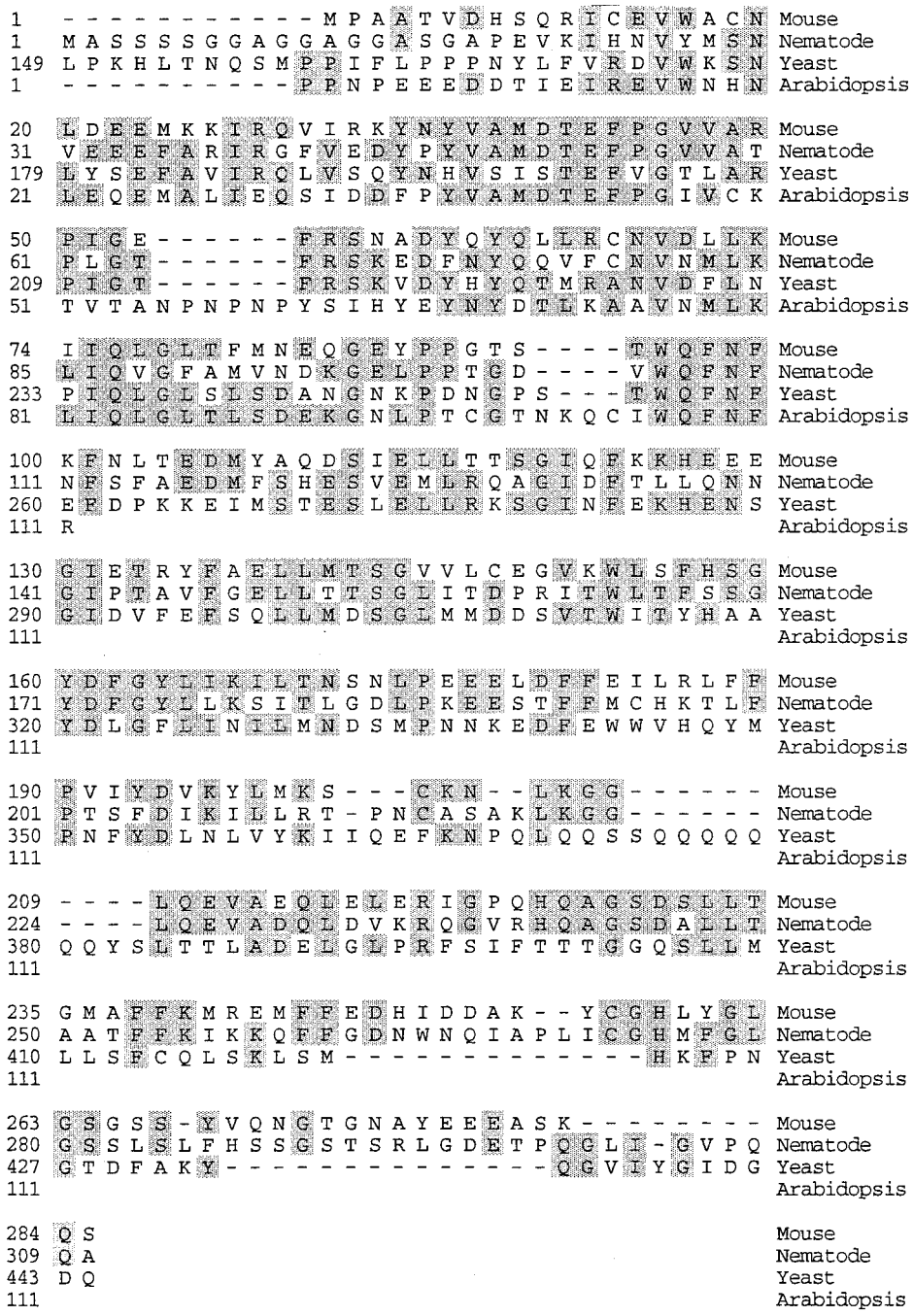

FIG. 3. Alignment of sequences of the yeast, mouse, *C. elegans*, and *A. thaliana* CAF1 proteins. Identical residues are shaded. The mCAF1 and cCAF1 sequences are given in their entireties. The *A. thaliana* CAF1 sequence represents the partial sequence available. Only the C-terminal 296 residues of yCAF1 (POP2) are shown.

was used to perform the CCR4 immunoprecipitations (data not shown). These data confirm that the interactions observed between CCR4 and mCAF1 proteins in the two-hybrid system result from an in vivo physical association of CCR4 with mCAF1.

The LRR of CCR4 is essential for the interaction of yCAF1 and mCAF1 with CCR4. To localize the CCR4 domain responsible for the interaction of CCR4 with yCAF1 (POP2) and mCAF1, we used various LexA-CCR4 derivatives in the two-hybrid system. All LexA-CCR4 derivatives were expressed to similar extents in yeast cells as determined by Western analysis (data not shown). As seen in Fig. 4, deletion of the N terminus (LexA-CCR4 Δ14-208) or truncation of the CCR4 protein (LexA-CCR4-1-668) reduced but did not eliminate the interaction of yCAF1 (POP2) and mCAF1 with CCR4, as judged by the increase in β-galactosidase activity over that of the corresponding LexA-CCR4 fusion with no GAL4 transactivation domain present. The smallest CCR4 fragment that we observed that was able to interact with CAF1 (POP2) was LexA-CCR4-302-837, which resulted in 35 U of β-galactosidase per mg in combination with an HA1-B42-yCAF1-85-441 fusion (data not shown). Small deletions within the LRR of CCR4, Δ391-435, Δ391-455, Δ218-394, completely abolished the inter-

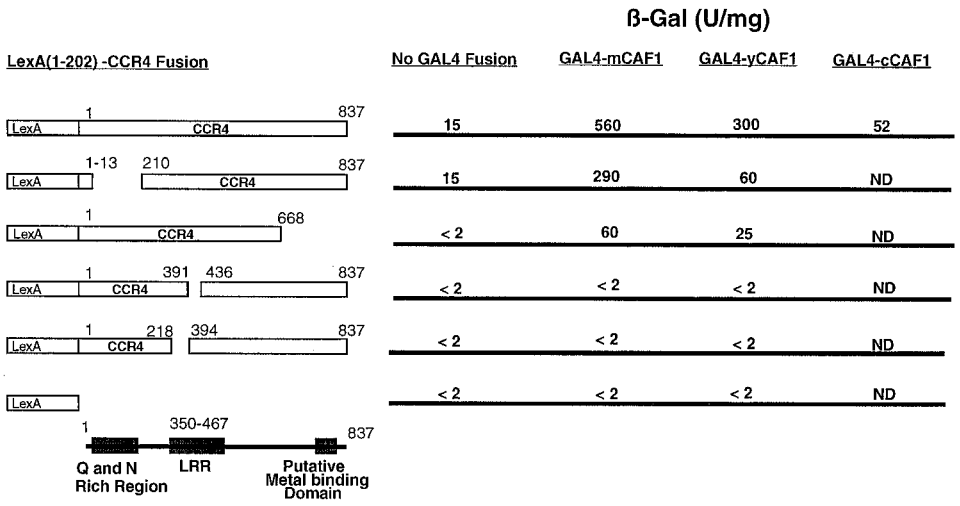

FIG. 4. Identification of the domain in CCR4 responsible for the interaction with CAF1. LexA-CCR4 derivatives were placed with GAL4 transactivation domain fusions of mCAF1, yCAF1, and cCAF1 in strain EGY188. All LexA-CCR4 derivatives were expressed at equivalent levels as judged by Western analysis (12) (data not shown). All strains carried the 34 reporter. Because of the weak nature of the interaction between CCR4 and cCAF1, we placed cCAF1 with only the LexA-CCR4-1-837 fusion. β-Galactosidase (β-Gal) assays represent the averages of at least three separate transformants. All standard errors of the means were less than 20%.

action of yCAF1 (POP2) or mCAF1 with CCR4 (Fig. 4). In support of the conclusion that the LRR of CCR4 is a domain important for the interaction of mCAF1 and yCAF1 (POP2) with CCR4, immunoprecipitation of proteins from a strain carrying LexA-CCR4 Δ391-435 and GAL4-mCAF1 by using an anti-LexA antibody failed to bring down the mCAF1 protein (Fig. 7, lane C). These results identify residues 302 to 668 of CCR4 as minimally required for interacting with CAF1 (POP2). However, unlike the binding of CCR4 to the 185- and 195-kDa species (12), we were not able to demonstrate that the LRR alone (residues 350 to 475) could interact with yCAF1 (POP2) or mCAF1 (data not shown). The LRR of CCR4 appears necessary but not sufficient for the association of mCAF1 and yCAF1 (POP2) with CCR4.

Disruption of the *CAF1* gene in yeast cells confers phenotypes similar to those conferred by disruptions of *CCR4*. Disruption of *CAF1* resulted in the inability to grow on rich medium containing glucose at 37°C as well as defects for growth in complete synthetic medium containing ethanol or glycerol as

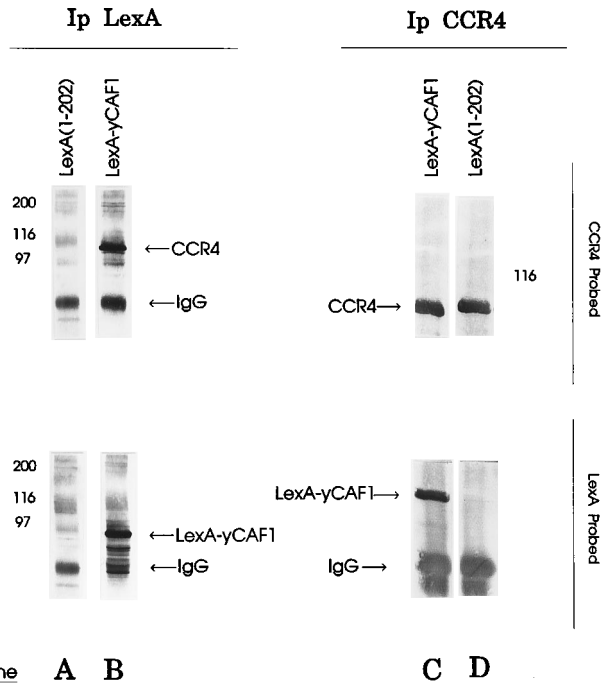

FIG. 5. yCAF1 immunoprecipitates with CCR4. Immunoprecipitations were conducted with strain EGY188 grown in 8% glucose. Conditions for immunoprecipitations are described in Materials and Methods. For lanes A and B, a LexA antibody was used to conduct the immunoprecipitations (LexA Ip); for lanes C and D, a CCR4 antibody was used to conduct the immunoprecipitations (CCR4 Ip). Proteins were visualized by Western analysis with either the antibody against CCR4 (CCR4 probed) or the antibody against LexA (LexA probed). IgG, immunoglobulin G. Sizes are indicated in kilodaltons.

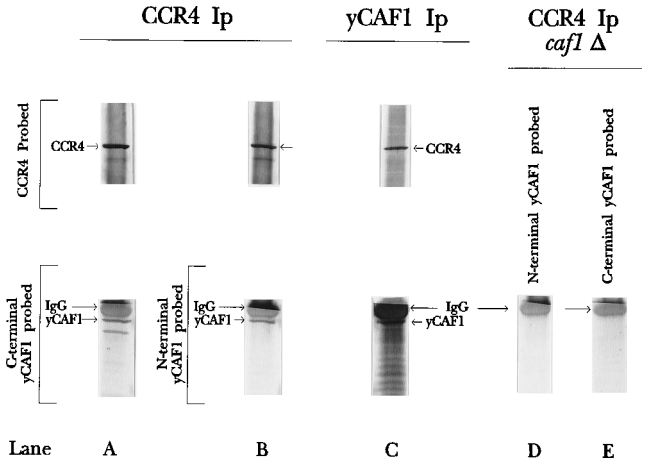

FIG. 6. Wild-type yCAF1 is associated with CCR4. Conditions for immunoprecipitations and Western blotting are described in Materials and Methods. For lanes A, B, D, and E, a CCR4 antibody was used to conduct the immunoprecipitations (CCR4 Ip); for lane C, a yCAF1 antibody directed against an N-terminal peptide of yCAF1 was used to conduct the immunoprecipitation (yCAF1 Ip). Lanes A to C, strain EGY191 (*CAF1*); lanes D and E, strain EGY191-2 (*caf1*). Proteins were visualized by Western analysis with either the CCR4 antibody (CCR4 probed) or the yCAF1 antibody (C terminal [lanes A and D] or N-terminal [lanes B, C, and E]) (yCAF1 probed). IgG, immunoglobulin G.

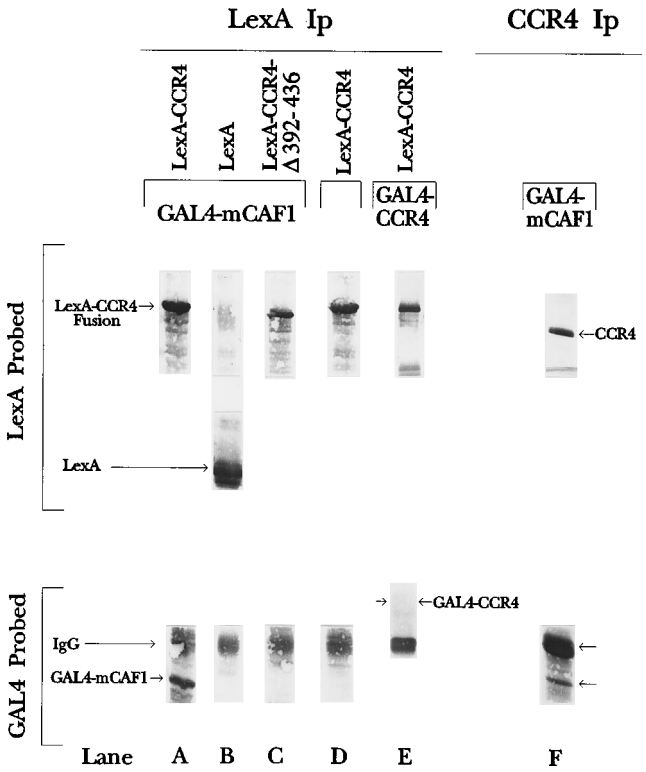

FIG. 7. mCAF1 coimmunoprecipitates with CCR4. Native immunoprecipitations were conducted as described in Materials and Methods from strains grown in 8% glucose. An antibody against LexA or CCR4 was used to immunoprecipitate the LexA-CCR4 fusions (LexA Ip) or wild-type CCR4 (CCR4 Ip), respectively. Western blots were probed with a GAL4 antibody (GAL4 probed), LexA antibody (LexA probed), or CCR4 antibody (lane F). Lanes A, B, and C, LexA immunoprecipitates from strains expressing LexA-CCR4, LexA, and LexA-CCR4 Δ392-436, respectively, along with GAL4-mCAF1; lane D, LexA-CCR4 immunoprecipitated from a strain expressing no GAL4 fusion; lane E, LexA-CCR4 immunoprecipitated from a strain expressing a GAL4-CCR4 fusion; lane F, CCR4 immunoprecipitated from a strain expressing the GAL4-mCAF1 fusion. The arrows in lane E indicate the place where GAL4-CCR4 protein would have been had it been present. IgG, immunoglobulin G.

TABLE 2. Effect on ADH II activity of a y*CAF1* disruption

Relevant genotype	Plasmid	ADH II activity (mU/mg)[a]	
		G	E
CAF1[b]		8	1,500
caf1[c]		5	760
ccr4-10 CAF1[d]		<1	420
ccr4-10 caf1[e]		5	160
spt10 CAF1[f]		44	2,300
spt10 caf1[f]		7	970
spt6 CAF1[g]		60	ND[h]
spt6 caf1[g]		6	ND
spt10 caf1[i]	LexA	18	ND
spt10 caf1[i]	LexA-yCAF1	77	ND
spt10 caf1[i]	LexA-yCAF1-1-181	30	ND
spt10 caf1[i]	LexA-yCAF1/mCAF1	56	ND

[a] ADH II activities were measured as described in Materials and Methods following growth on either glucose (G)- or ethanol (E)-containing YEP medium except for strains containing plasmids, which were grown on minimal medium. Values represent the average of at least three determinations for individual strains, the average of at least four different strains in the case of segregants, and the average of at least four different transformants for strains containing plasmids. The SEMs (standard errors of means) were less than 15% except for values less than 10, whose SEMs were less than 30%. For the strains containing LexA-yCAF1-1-181 and LexA-yCAF1-1-181/mCAF1, the SEMs were 12 and 6%, respectively, indicating that differences observed between these two strains were highly significant. In comparing ADH II activities that are both less than 10 mU/mg, any differences are not considered significant.

[b] Strain 935-2.

[c] Strain 935-2-3.

[d] Strain 935-1.

[e] Strain 935-1-6.

[f] Segregants from cross 992-6a × 994-2.

[g] Segregants from cross 147-6d × 935-2-3.

[h] ND, not done.

[i] Strain 1005-2-3c with the plasmids indicated, as depicted in Fig. 8.

the sole carbon source as were previously observed for defects in *POP2* (25). The phenotypic defect for growth on a nonfermentative medium is similar to that observed for strains with *ccr4* disruptions, which exhibit temperature sensitivity at 37°C when a nonfermentative carbon source is used. A *caf1* (*pop2*) strain also displayed a twofold reduction in ADH II activity under derepressed conditions compared with an isogenic strain containing the wild-type *CAF1* (*POP2*) allele (Table 2). This effect is similar to the fivefold decrease in *ADH2* expression caused by a *ccr4* allele (10). Most importantly, disruption of *CAF1* (*POP2*) suppressed the increased *ADH2* expression under glucose growth conditions that is observed in an *spt6* or *spt10* mutant background (Table 2). CCR4 is the only other known suppressor of the *spt6* or *spt10* defects. LexA-yCAF1 was able to complement the *caf1* (*pop2*) disruption in an *spt10* background and give rise to increased *ADH2* expression (Table 2). LexA-yCAF1 had no effect on ADH II enzyme levels in strains that were not mutated for *spt10* or that contained the *caf1* allele alone (data not shown). The N-terminal 181 residues of yCAF1 were able to provide only a slight complementation of the *caf1* defect in a *caf1 spt10* background (Table 2). In contrast, LexA-yCAF1/mCAF1, which substitutes the entire mCAF1 sequence for the 182 to 444 C-terminal residues of yCAF1, resulted in a much greater increase of *ADH2* expression in the *caf1 spt10* strain (Table 2). LexA-yCAF1 could also complement the *caf1* temperature-sensitive phenotype. Deletion of an N-terminal region of yCAF1 (LexA-yCAF1-80-444; Fig. 8) resulted in a protein that was unable to complement the 37°C phenotype (data not shown). Neither LexA-yCAF1/mCAF1 nor LexA-yCAF1/cCAF1 could complement the *caf1* (*pop2*) temperature-sensitive phenotype (data not shown).

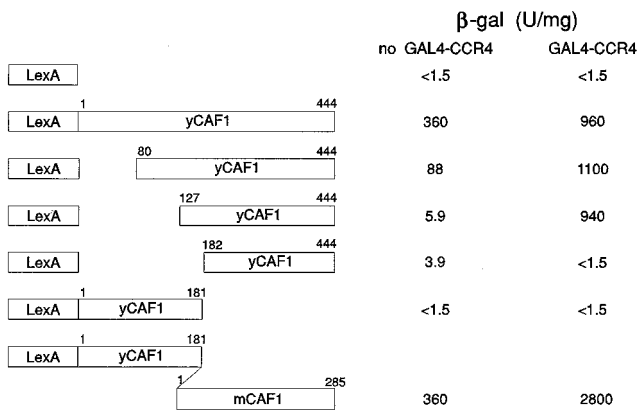

FIG. 8. LexA-yCAF1 can function as a transcriptional activator. β-Galactosidase (β-gal) assays and interactions with GAL4-CCR4 were conducted as described in Materials and Methods and in the legends to Fig. 2 and 4. Amino acid residues of each segment of the CAF1 protein are indicated. All assays and interactions were done in strain EGY188 with the 34 reporter. All LexA-CAF1 derivatives were expressed at equivalent levels as assessed by Western analysis (data not shown).

TABLE 3. *CAF1* is required for maximal activation potential of LexA activators[a]

LexA fusion	Relevant genotype	β-Galactosidase activity (U/mg)
LexA-CCR4-1-837	*CAF1*	15
LexA-CCR4-1-837	*caf1*	4.6
LexA-CCR4-1-160	*CAF1*	1,000
LexA-CCR4-1-160	*caf1*	230
LexA-CCR4-1-13/210-302	*CAF1*	2,300
LexA-CCR4-1-13/210-302	*caf1*	960
LexA-ADR1-1-1323	*CAF1*	940
LexA-ADR1-1-1323	*caf1*	420
LexA-ADR1-147-359 (TADII)	*CAF1*	150
LexA-ADR1-147-359 (TADII)	*caf1*	32
LexA-ADR1-359-737 (TADIII)	*CAF1*	320
LexA-ADR1-359-737 (TADIII)	*caf1*	99
LexA-B42	*CAF1*	960
LexA-B42	*caf1*	280
LexA-yCAF1	*CCR4*[b]	210
LexA-yCAF1	*ccr4*[c]	64
LexA-yCAF1/mCAF1	*CCR4*[b]	780
LexA-yCAF1/mCAF1	*ccr4*[c]	180
LexA	*CCR4*[b]	<1.5
LexA	*ccr4*[c]	<1.5

[a] β-Galactosidase activities were determined as described in Materials and Methods. The 34 reporter was used in all cases except LexA-ADR1-1-1323 and LexA-ADR1-359-737, in which cases the 1840 reporter (one LexA operator site) was used. All standard errors of the means were less than 20%. Strains EGY191 (*CAF1*) and EGY191-2 (*caf1*) are isogenic except for the *CAF1* allele. The activity of LexA alone with the 34 or 1840 reporter in strain EGY191 or EGY191-2 was less than 1.5.

[b] Strain MD9-7c+.

[c] Strain MD9-7c.

yCAF1 is necessary for full activation by LexA hybrid activators. We have previously shown that the *CCR4* gene is required for maximal transcriptional activation by LexA-fused transactivator proteins (12). We therefore examined whether disruptions of *CAF1* (*POP2*) could also affect the ability of LexA-bound activators to function in yeast cells. The results shown in Table 3 indicate that the *caf1* (*pop2*) allele resulted in a two- to fivefold reduction in the activation potential of several different activators tested. This reduction occurred for weak activators like full-length CCR4 or TADII from ADR1 and was observed for stronger activators like B42, full-length ADR1, TADIII of ADR1, or the individual CCR4 activation domains which exhibit strong activation potential when removed from the CCR4 C-terminal sequences (12). yCAF1, like CCR4, appears important for full activation potential but does not appear to be essential for any particular type of activator.

yCAF1 and mCAF1 can activate transcription when fused to LexA. Fusion of CCR4 to LexA can activate transcription from a LexA operator-controlled reporter gene (12). LexA-yCAF1-1-444 also was capable of transactivation (Fig. 8). Unlike CCR4, however, the activation potential of CAF1 (POP2) showed no carbon source regulation (data not shown) (12). Both the 80 to 127 region in addition to the C-terminal CCR4-binding region of CAF1 (POP2) appeared required for transactivation (Fig. 8). The transcriptional activity of LexA-yCAF1 was reduced threefold, but not eliminated, by a *ccr4* disruption (Table 3), indicating that the ability of yCAF1 to recruit the transcriptional machinery is not solely dependent on CCR4. Substitution of mCAF1 sequences for that of the C-terminal region of yCAF1 (residues 182 to 444) resulted in a transcriptionally active yeast-mouse CAF1 protein (Fig. 8). This LexA-yCAF1/mCAF1 protein retained its ability to activate transcription in a *ccr4-10* background (Table 3), indicating that LexA-yCAF1/mCAF1 activation ability was not the result of binding to CCR4. The reason why LexA-yCAF1/mCAF1 displayed a much higher activation ability in strain MD9-7c+ (Table 3) than it did in strain EGY188 (Fig. 8) is not clear. Western analysis indicated that there were comparable levels of the protein in the two different strains (data not shown). LexA-yCAF1-1-181, which was incapable of activating transcription (Fig. 8), remained transcriptionally inactive in the *ccr4-10* background (<1 U of β-galactosidase per ml; data not shown). A LexA-yCAF1/cCAF1 hybrid was transcriptionally inactive (data not shown). The ability of each of these LexA-CAF1 fusions to bind CCR4 in the two-hybrid system was also monitored. As expected, LexA-yCAF1-127-444 was able to bind GAL4-CCR4, but the shorter LexA-yCAF1-182-444 was not (Fig. 8). In addition, LexA-yCAF1/mCAF1 again interacted better with GAL4-CCR4 than did LexA-yCAF1 (compare with results in Fig. 4).

DISCUSSION

CAF1 is an evolutionarily conserved protein that associates with CCR4. We have identified a mouse protein which is capable of interacting and binding to the yeast CCR4 regulatory complex. The mCAF1 protein was shown to have a high degree of similarity to proteins from humans, *C. elegans*, *A. thaliana*, and *S. cerevisiae*. Since the CAF1 sequences were derived from mammalian tissues at different developmental stages and from vastly different eukaryotic organisms, we believe that the CAF1 protein is an important regulatory component ubiquitous to all cell types and all eukaryotic organisms. The yCAF1 protein contained an extra 148 amino acids at the N-terminal region lacking from the other homologs. This region, while not required for binding CCR4, was important for the function of the yCAF1 (POP2) protein in complementing a *caf1* (*pop2*) allele and in activating transcription when fused to LexA. This N-terminal region also produced a slight rescue of the *caf1* (*pop2*) suppression of an *spt10* allele (Table 2). The N terminus of yCAF1 would appear, therefore, to make a species-specific contact in yeast cells. The observations that mCAF1, cCAF1, and the C-terminal region of yCAF1 can each bind to or interact with CCR4 indicates that the C-terminal segment of CAF1 has retained an evolutionarily conserved function. It is likely, therefore, that CCR4 also exists in higher eukaryotes.

The evolutionary conservation of function between mCAF1 and yCAF1 extends beyond the binding to CCR4. LexA-yCAF1 fusions were by themselves able to act as transcriptional activators. This activity was reduced but not eliminated by a *ccr4* allele, indicating that yCAF1 (POP2) could recruit the transcriptional machinery independently of CCR4. mCAF1 could also functionally substitute for the C-terminal region of yCAF1 (POP2) in the in vivo transcription assay. The transactivation ability of mCAF1 was separate from its binding to CCR4 since LexA-yCAF1/mCAF1 remained transcriptionally active in a *ccr4*-containing strain. Moreover, both LexA-yCAF1 and the LexA-yCAF1/mCAF1 hybrid could complement a *caf1* disruption in its suppression of *spt10*-induced *ADH2* expression. Thus, LexA-yCAF1/mCAF1 is able to form functional complexes in yeast cells. The inability of LexA-yCAF1/mCAF1 to complement the 37°C temperature-sensitive growth phenotype of a *caf1* (*pop2*) disruption suggests that at least one contact that yCAF1 makes has not been conserved with mCAF1. cCAF1, in contrast to mCAF1, was neither transcriptionally active in yeast cells nor, as best as we could determine, able to form stable immunoprecipitable complexes with CCR4.

CAF1 is required for diverse transcriptional processes. Dis-

ruption of *CAF1* (*POP2*) gave phenotypes similar to those seen with disruptions of *CCR4*. A *caf1* allele affected the ability of the *ADH2* gene to derepress, eliminated the increased ADH II activity observed with mutations in *SPT6* or *SPT10*, and caused a general defect in the ability to utilize nonfermentative carbon sources (25). *pop2* (*caf1*) was also shown to reduce isocitrate lyase and invertase derepression (25), and *CCR4* has been shown previously to be required for isocitrate lyase derepression (7). However, a *caf1* (*pop2*) disruption resulted in a temperature-sensitive defect under glucose growth conditions, a phenotype not consistently observed with a *ccr4* deletion (unpublished observations). In an obverse relationship, *ccr4* disruptions resulted in a cold-sensitive phenotype that has not been observed with a *caf1* (*pop2*) disruption. We interpret these results as evidence that CAF1 (POP2) is part of the CCR4-containing complex but that its function may be somewhat different from that of CCR4. In addition, we observed that CAF1 (POP2) was required for maximal LexA activator-induced transcription. These results suggest that CAF1 (POP2), like CCR4, is a transcription factor required for a diverse set of genes or processes, only some of which are involved in or related to carbon metabolism.

Both *CAF1* (*POP2*) and *CCR4* appear not only to be required for transcriptional processes but in some cases to act as negative regulators of gene expression. Deletion of *POP2* (*CAF1*) was shown to greatly augment *PGK* expression during stationary phase (25). CCR4 was similarly suggested to negatively regulate the *MET3* gene (20). Recently, it has also been reported that *CCR4* and *CAF1* mutations suppress the defects of a *rad52-20* allele, possibly by elevating *rad52-20* gene expression (27). SPT10 has also been shown to both repress and activate transcription (21). It is possible that the ability of CCR4 and CAF1 (POP2) to both enhance and restrain gene expression results from gene-specific contacts that this complex makes.

While the specific functions of CCR4, CAF1 (POP2), and their associated factors are unclear, the CCR4 protein complex seems distinct from several other transcriptionally important complexes. CCR4 does not coimmunoprecipitate with SPT6 or SPT10 (9), and CCR4 is not part of the SNF/SWI protein complex (9) that is important to allowing activator accessibility to nucleosomes (6). CCR4 and CAF1 also have not been found in the yeast holoenzyme or mediator complexes (3a). The CCR4/CAF1 complex appears, therefore, to play a unique role in gene regulation. Further characterization of the CCR4 complex and its interaction with transcriptional components will shed light on the function of the evolutionarily conserved CAF1 proteins and CCR4.

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