DBF2, a cell cycle-regulated protein kinase, is physically and functionally associated with the CCR4 transcriptional regulatory complex

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CCR4, a general transcriptional regulator affecting the expression of a number of genes in yeast, forms a multi-subunit complex in vivo. Using the yeast two-hybrid screen, we have identified DBF2, a cell cycle-regulated protein kinase, as a CCR4-associated protein. DBF2 is required for cell cycle progression at the telophase to G1 cell cycle transition. DBF2 co-immunoprecipitated with CCR4 and CAF1/POP2, a CCR4-associated factor, and co-purified with the CCR4 complex. Moreover, a dbf2 disruption resulted in phenotypes and transcriptional defects similar to those observed in strains deficient for CCR4 or CAF1. ccr4 and caf1 mutations, on the other hand, were found to affect cell cycle progression in a manner similar to that observed for dbf2 defects. These data indicate that DBF2 is involved in the control of gene expression and suggest that the CCR4 complex regulates transcription during the late mitotic part of the cell cycle.

Keywords: CCR4/cell cycle/DBF2/protein kinase/transcription

Introduction

The CCR4 protein from Saccharomyces cerevisiae affects the expression of a number of genes and processes. CCR4 is required for full derepression of ADH2 and other non-fermentative genes under glucose-derepressed conditions (Denis, 1984; Denis and Malvar, 1990). ccr4 mutations also reduce the enhanced gene expression resulting from defects in the SPT6 or SPT10 proteins (Denis, 1984; Denis and Malvar, 1990) that appear important in maintaining a proper chromatin structure (Natsoulis et al., 1991; Dollard et al., 1994; Bortvin and Winston, 1996). CCR4 functions downstream of SPT6 and SPT10, at a post-chromatin remodeling event (Denis et al., 1994; M.Caserta, personal communication). In addition to affecting these processes, a ccr4 allele affects the expression of genes involved in cell wall integrity (A.Sakai, personal communication), in UV sensitivity (Schild, 1995) and in methionine biosynthesis (McKenzie et al., 1993). Moreover, CCR4 is required by different transactivators to function maximally (Draper et al., 1994). These observations indicate that CCR4 plays an important general transcriptional role in diverse cellular events.

CCR4 is a leucine-rich repeat (LRR)-containing protein (Malvar et al., 1992). Proteins containing LRRs have often been found to be associated with other proteins through their LRR region (Kobe and Diesenhofer, 1994). CCR4 is a component of a multi-subunit complex, and the LRR region is essential for its protein–protein interactions in this complex (Draper et al., 1994, 1995). The CAF1/POP2 protein has been identified as a component of the CCR4 complex (Draper et al., 1995), and caf1 disruptions display very similar phenotypes and transcriptional defects to those of ccr4 (Šakai et al., 1992; Draper et al., 1995).

We report here that DBF2 is another component of the CCR4 complex. DBF2 was identified as a temperature-sensitive mutation that causes cell cycle arrest at the end of mitosis in which the cells have a fully extended spindle and divided chromatin, a characteristic of telophase (Toyn et al., 1994). Despite there being temperature-sensitive alleles of DBF2, deletions of the gene are viable (Toyn et al., 1997) due to the existence of a homolog, DBF20 (Toyn et al., 1991). However, deletion of both DBF2 and DBF20 results in strains that are non-viable, indicating that these genes encode closely related protein kinases that are essential for the ending of mitosis. The target protein substrates of the DBF2 kinase have not been identified, and, therefore, the molecular basis for its role in regulation of the cell cycle is not known. Our present work indicates a role for DBF2 in transcriptional regulation. We find that a defect in DBF2 results in phenotypes and transcriptional defects similar to those observed for a ccr4 or caf1 disruption. Conversely, ccr4 and caf1 disruptions affect cell cycle progression in late mitosis similarly to dbf2 mutations. The CCR4 complex appears, therefore, to be important to the control of specific sets of genes, including those involved in the late mitotic phase of the cell cycle.

Results

DBF2 associates with CCR4 and CAF1

To identify further members of the CCR4 and CAF1 complex, a yeast two-hybrid screen was carried out using the LexA–CCR4 fusion protein as the bait (Draper et al., 1995). The interaction library contained the Escherichia coli-derived B42 activator fused to yeast genomic DNA fragments under the control of a GAL1 promoter (Zervos et al., 1993). Fifty six colonies that displayed galactose-
Table 1. Two-hybrid interactions of DBF2 with CCR4 and CAF1

<table>
<thead>
<tr>
<th>LexA fusion</th>
<th>B42 fusion</th>
<th>β-Gal (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA–CCR4</td>
<td>B42</td>
<td>30</td>
</tr>
<tr>
<td>LexA–CCR4</td>
<td>B42–DBF2 (205–561)</td>
<td>150</td>
</tr>
<tr>
<td>LexA–CCR4</td>
<td>B42–DBF2 (1–561)</td>
<td>170</td>
</tr>
<tr>
<td>LexA–CCR4</td>
<td>B42–DBF2 (K195T)</td>
<td>110</td>
</tr>
<tr>
<td>LexA</td>
<td>B42–DBF2 (205–561)</td>
<td>1.3</td>
</tr>
<tr>
<td>LexA–CCR4 (ΔLRR)</td>
<td>B42–DBF2 (205–561)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>LexA–CAF1</td>
<td>B42</td>
<td>110</td>
</tr>
<tr>
<td>LexA–CAF1</td>
<td>B42–DBF2 (205–561)</td>
<td>210</td>
</tr>
<tr>
<td>LexA–CAF1</td>
<td>B42–DBF2 (1–561)</td>
<td>1700</td>
</tr>
<tr>
<td>LexA–CAF1</td>
<td>B42–DBF2 (K195T)</td>
<td>1500</td>
</tr>
</tbody>
</table>

Plasmids that directed the synthesis of LexA fusion proteins were introduced into strain EGY188 containing the LexA–lacZ reporter p34 that contains eight LexA-binding sites upstream of LacZ. β-Galactosidase activities represent averages of at least three separate transformants. Standard error of measurements (SEMs) was <20% in each case. Strains were grown on minimal medium lacking uracil, histidine and tryptophan and supplemented with 2% raffinose and 2% galactose as previously described (Draper et al., 1995).

DBF2 is physically associated with the CCR4 complex

The physical association of B42–DBF2 with CCR4 was examined by co-immunoprecipitation. Whole-cell extract expressing the B42–DBF2 (full-length) fusion protein containing an HA1 tag was incubated with CCR4 antibody. The immunoprecipitated samples were analyzed by Western blotting using antibody directed against CCR4 and HA1 antibodies. The immunoprecipitated samples were subjected to electrophoresis on a 10% SDS–PAGE gel. CCR4- and HA1-containing proteins were detected by Western analysis as described (Draper et al., 1995). Lanes 1 and 2, crude extracts containing B42–SIP1 and B42–DBF2, respectively; lane 3, B42–SIP1-containing extracts treated with anti-CCR4 antibody; lane 4, same as lane 3 except B42–DBF2; lane 5, same as lane 4 except extracts were treated with anti-LexA antibody. (B) Extracts containing B42–DBF2 were immunoprecipitated and analyzed as described in (A) above. Lane 1, crude extract from EGY188-1 (ccr4Δ); lane 2, crude extract from EGY188-1 (caff1); lane 3, crude extract from EGY188 (wt); lanes 4 and 5, CCR4 immunoprecipitations using strains EGY188-1 and EGY188, respectively; lanes 6 and 7, CAF1 immunoprecipitations using strains EGY188-1 and EGY188, respectively. Lanes 1–3 were developed with anti-HA1 antibody whereas lanes 4–7 were developed with both anti-CCR4 and anti-HA1 antibodies. For clarity, lanes 4–7 were not treated with anti-CAF1 antibody since other experiments have shown that CCR4 and CAF1 always co-immunoprecipitate (Draper et al., 1995; data not shown).
that B42–DBF2 interacts with CCR4 specifically via the DBF2 moiety. We also showed that B42–DBF2 was also co-immunoprecipitated with CAF1 using an anti-CAF1 antibody (Figure 1B, lane 7) and was not immunoprecipitated from extracts prepared from a strain lacking CAF1 protein (lane 6). We were not able, however, to co-immunoprecipitate CCR4 or CAF1 with B42–DBF2 in strains deleted for caf1 or ccr4, respectively.

We further examined the physical interaction between DBF2 and CCR4 using a second approach. We have found that CCR4 and a CAF1-6His-tagged protein co-purify following two chromatographic stages: Ni²⁺-NTA agarose and Mono-Q chromatography (Figure 2A). The CAF1-6His gene, containing one copy of a CAF1 gene fused at its 3' end to a six histidine tag and integrated into the yeast genome at the TRP1 locus, was able to complement phenotypes associated with a caf1 null allele (data not shown). We subsequently used a LexA–DBF2 construct was capable of complementing a dbf2 null allele (data not shown) to analyze the co-purification of DBF2 with CAF1. Whole-cell extract prepared from a strain expressing both LexA–DBF2 and CAF1-6His was first passed over an Ni²⁺-NTA–agarose column and the bound proteins were eluted with imidazole. The resulting eluant was then passed over an FPLC Mono-Q column, and the bound proteins were eluted with a linear salt gradient. The resulting Mono-Q fractions were subjected to Western blot analyses (Figure 2B). The LexA–DBF2 fusion protein co-purified with CAF1 through the Ni²⁺-NTA and Mono-Q columns (Figure 2B). In a control experiment, LexA alone was not retained on the Ni²⁺-agarose column (Figure 2C, lane 3, LexA, compared with lane 4, LexA–DBF2), indicating that it is the DBF2 moiety of LexA–DBF2 which is co-purifying with CAF1-6His. The co-purification experiment together with the co-immunoprecipitation experiments clearly indicate that DBF2 is associated with the CCR4 complex in vivo.

The CCR4 complex displays DBF2-dependent protein kinase activity

Since DBF2 encodes a protein kinase, we addressed the question as to whether the CCR4 complex contained a protein kinase by using an in vitro kinase assay. Galactose-grown extracts prepared from a strain expressing the B42–DBF2 fusion (containing the HA1 epitope and under the control of the galactose-inducible GAL1 promoter) were immunoprecipitated with HA1, CCR4 or LexA control antibody, and the resulting immunoprecipitates were then analyzed for the ability to phosphorylate H1 histone (Figure 3A). Both anti-HA1 and anti-CCR4 immunoprecipitates displayed protein kinase activity (lanes 1 and 3). No protein corresponding to H1 histone was phosphorylated when H1 histone was left out of the reaction (lane 2). In control experiments, both the immunoprecipitates obtained with LexA antibody (lane 4) and extracts incubated with protein A–agarose alone (data not shown) contained much less kinase activity than observed with the anti-CCR4 antibody immunoprecipitates (Figure 3A, compare lane 4 with lane 3). These results indicate that the CCR4 immunoprecipitates contain protein kinase activity. To examine whether the kinase activity in the CCR4 complex is due to the presence of DBF2, we repeated the kinase experiment by immunoprecipitating the CCR4 complex from extracts of either glucose-grown or galactose-grown cultures. Protein kinase activity in anti-CCR4 antibody immunoprecipitates observed in the galactose-inducing condition (Figure 3B, lane 4) was much greater than that in the glucose-repressing condition (lane 2). Under galactose growth conditions with LexA
Fig. 3. CCR4 immunoprecipitates contain DBF2 protein kinase activity. (A) Extracts from strain MLF6 containing B42–DBF2 (lanes 1–4) grown under galactose growth conditions were first treated with antibody and then the immunoprecipitates were analyzed for H1 histone protein kinase activity, as described, following SDS–PAGE and fluorography (Toyn and Johnston, 1994). The presence of added H1 histone is indicated above the autoradiograms. Lane 1, immunoprecipitation was conducted with HA1 epitope antibody; lanes 2 and 3, immunoprecipitations were conducted with anti-CCR4 antibody; lane 4, same as lane 3 except LexA antibody. (B) Protein kinase assays were conducted as described in (A) except that strain MLF6 containing B42–DBF2 was grown under glucose or galactose growth conditions as indicated. Immunoprecipitations as indicated were conducted as described in (A). (C) Extracts from strain MFL6 containing B42–DBF2 (lanes 2 and 4) or B42 (lanes 1 and 3) grown under galactose growth conditions were assayed for kinase activity as described in (A) above. Lanes 1 and 2, immunoprecipitations were conducted with anti-CCR4 antibody; lanes 3 and 4, immunoprecipitations were conducted with HA1 epitope antibody. (D) Re-immunoprecipitation of extracts following the first immunoprecipitation protein kinase assay was conducted as described in Materials and methods. Lane 1, the first immunoprecipitation was conducted with anti-CCR4 antibody and the second with CCR4 antibody; lane 2, same as lane 1 except that the second immunoprecipitation was conducted with CAF1 antibody; lane 3, same as lane 1 except the second immunoprecipitation was conducted with HA1 antibody; lane 4, same as lane 3 except HA1 antibody was used for both immunoprecipitations.

imunoprecipitates (lane 3), protein kinase activity was much less than observed for the CCR4 immunoprecipitates (Figure 3B, lane 4). This background level of protein kinase activity was also observed in anti-CCR4 antibody immunoprecipitates of extracts from a ccr4-deleted strain (data not shown). Moreover, under galactose growth conditions, increased protein kinase activity was observed for B42–DBF2-containing strains in both the CCR4 (Figure 3C, lane 2) and HA1 (lane 4) immunoprecipitates as compared with comparable immunoprecipitates obtained from B42-only expressing strains (see lanes 1 and 3, respectively). These results indicate that the increased protein kinase activity observed under galactose conditions from CCR4 immunoprecipitates was indeed due to the expression of the B42–DBF2 fusion protein.

To test if any of the known components in the CCR4 immunoprecipitates can be phosphorylated by DBF2, we re-immunoprecipitated CCR4, CAF1 or B42–DBF2 following the kinase assay. First, the CCR4 complex was immunoprecipitated with the CCR4 antibody and the B42–DBF2 fusion protein was immunoprecipitated with the HA1 antibody. The resulting immunoprecipitates were then subjected to the in vitro kinase assay. CCR4, CAF1 and B42–DBF2 subsequently were re-immunoprecipitated out of the CCR4 immunoprecipitates by adding, respectively, the CCR4, CAF1 and HA1 antibodies, and B42–DBF2 was re-immunoprecipitated from the HA1 immunoprecipitates by adding HA1 antibody again. The resulting immunoprecipitates were subjected to SDS–PAGE and fluorography (Figure 3D). Phosphorylated B42–DBF2 was identified in the HA1 double immunoprecipitation (lane 4) and from the CCR4 immunoprecipitate that was re-treated with HA1 antibody (lane 3). Phosphorylation of CCR4 or CAF1 was not observed from the CCR4/CCR4 and CCR4/CAF1 double immunoprecipitates (lanes 1 and 2), respectively). Separate experiments showed that CCR4 or CAF1 could be detected by Western analysis from these re-immunoprecipitated extracts (data not shown). These data suggest that neither CCR4 nor CAF1 is an in vitro target for DBF2.

The initial two-hybrid interaction between CCR4 and DBF2 indicated that the DBF2 kinase domain was not required for interaction with CCR4, although it was required for interaction with CAF1 (Table I). To determine specifically if DBF2 kinase function was required for its interaction with CCR4 or CAF1, a DBF2 allele containing a mutation (K195T) in the conserved lysine residue of the ATP-binding site catalytic domain of DBF2 was analyzed (Toyn and Johnston, 1993). This dbf2-K195T allele does not complement the dbf2 null allele, is lethal in the absence of the wild-type DBF2 gene and lacks kinase activity (Toyn and Johnston, 1994). B42–DBF2-K195T interacted with both CCR4 and CAF1 to nearly the same extent as did the wild-type B42–DBF2 protein (Table I). These experiments indicate that DBF2 protein kinase activity is not important for DBF2 association with CCR4 or CAF1, and that CCR4 and CAF1 are not in vitro substrates for DBF2 under the conditions utilized.

A dbf2 disruption causes transcriptional defects similar to those observed with disruption of ccr4 and caf1

Disruption of CCR4 or CAF1 results in a number of transcriptional phenotypes. We investigated the putative
role of DBF2 in several of these transcriptional processes. ccr4 mutations were identified originally as specific suppressors of the enhanced ADH2 expression under glucose growth conditions caused by an spt10 defect (Denis, 1984; see Table II). Only ccr4 and caf1 alleles display this phenotype (Draper et al., 1995). A dbf2 disruption, which grows almost as well as the wild-type, similarly suppressed the enhanced ADH2 enzyme levels caused by an spt10 defect (Table II). The dbf2 effect was specific to suppressing spt10-enhanced ADH2 expression, since dbf2 was incapable of suppressing an ADRI-5® allele which displays high ADH2 enzyme levels under repressed conditions similar to those observed in an spt10-containing strain. In contrast, a deletion of DBF2, a non-cell cycle-controlled homolog of DBF2, had no effect on spt10-enhanced ADH2 expression (Table II).

crr4 and caf1 mutations also cause a defect in non-fermentative growth at 37°C, a phenotype we observed to be shared by dbf2 (Table III, see glyceral column). This arrest of growth on glycerol by dbf2 was not a mitotic arrest as is observed with temperature-sensitive alleles of DBF2 (data not shown). While a ccr4 disruption affects the non-fermentative expression of ADH2 by 5-fold (Denis and Malvar, 1990) and caf1 by ~2-fold (Draper et al., 1995), a dbf2 effect on ADH2 expression under non-fermentative growth conditions was not observed (data not shown). caf1 and ccr4 defects also increase sensitivity to staurosporine, a protein kinase inhibitor (A.Sakai, personal communication) and to caffeine, two compounds linked to effects on cell wall integrity and other processes (Posas et al., 1993; Costigan et al., 1994) (Table III). These effects are similar to those observed with defects in the protein kinase C and MAP kinase pathway (Lee et al., 1993; Costigan et al., 1994) and appear to result from defects in expression of genes involved in cell wall integrity (Roemer et al., 1994; Shimizu et al., 1994). A dbf2 disruption resulted in similar increased sensitivity to caffeine and staurosporine (Table III). The staurosporine sensitivity and temperature sensitivity of dbf2, caf1 and ccr4 deletions were reversed by 1 M sorbitol as well as the cold sensitivity and temperature sensitivity phenotypes of these disrupted alleles (Table III) (A.Sakai, personal communication). We also observed that ccr4- (B.Anderson, personal communication), caf1- and dbf2-containing strains were hypersensitive to elevated levels of the Li⁺ ion (Table III). It should be noted that combining the dbf2 disruption with either a ccr4 or caf1 disruption resulted in no increased sensitivity of any of the above phenotypes, suggesting that DBF2 functions in the same pathway as CCR4 and CAF1.

In addition, we examined the role of DBF2 in affecting LexA transactivator function. Both ccr4 and caf1 defects reduce the ability of several different LexA activators to activate a LexA–lacZ reporter ~2- to 3-fold (Draper et al., 1994, 1995). A dbf2 disruption affected the function of LexA–B42, LexA–ADR1-TADIV and LexA–CCR4–1–fermentative growth conditions was not observed (data not shown). caf1 and ccr4 defects also cause increased sensitivity to staurosporine, a protein kinase inhibitor (A.Sakai, personal communication) and to caffeine, two compounds linked to effects on cell wall integrity and

**Table II. dbf2 suppresses spt10-enhanced expression.**

| spt10⁰ | 87 |
| dbf2/spt10⁰ | 9.0 |
| ADRI-5° | 100 |
| dbf2 ADRI-5° | 120 |
| spt10⁰ | 210 |
| dbf2/spt10⁰ | 200 |
| ccr4/spt10⁰ | 175 |
| ADH II (mU/mg) | 23 |

ADH II enzyme activities were assayed following growth on medium containing 8% glucose as described in Materials and methods. SEMs were <20%. Values represent the average of at least three segregants.

**Table III. dbf2, caf1 and ccr4 phenotypes.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>12°C</th>
<th>12°C + sorb.</th>
<th>37°C</th>
<th>37°C + sorb.</th>
<th>Gly 37°C</th>
<th>Stauro + sorb.</th>
<th>Caffeine</th>
<th>LiCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>188</td>
<td>wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>188-1</td>
<td>ccr4</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>188-1c</td>
<td>caf1</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>S7-4A</td>
<td>dbf2</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
</tr>
</tbody>
</table>

Growth phenotypes were determined on YEP plates containing 2% glucose as the carbon source except as indicated. Stauro. plates contained 2 mM staurosporine; sorb. plates contained 1 M sorbitol; Gly = YEP plates containing 3% glycerol; caffeine plates were supplemented with 8 mM caffeine; LiCl plates were supplemented with 0.3 M LiCl. The designation ‘+_’ refers to good growth, ‘w’ to minimal growth, and ‘–’ to no growth. Growth in strain S7-4A was compared with its isogenic wild-type parent S7-4A-cmyc which behaved similarly to strain EGY188.
Table IV. Cells with divided chromatin in caf1 and ccr4 cultures

<table>
<thead>
<tr>
<th>Yeast strains</th>
<th>Buds (%)</th>
<th>Divided chromatin (%)</th>
<th>Budded cells with divided chromatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>62</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>ccr4Δ</td>
<td>75</td>
<td>44</td>
<td>57</td>
</tr>
<tr>
<td>wtΔ</td>
<td>45</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>caf1Δ</td>
<td>54</td>
<td>20</td>
<td>37</td>
</tr>
</tbody>
</table>

Log phase cultures were grown in YEP medium plus glucose at 30°C and stained with DAPI. Two hundred cells of each culture (as indicated) were observed by fluorescence microscopy. Cells with divided chromatin had elongated spindles similar to those observed with dbf2-containing strains (Toyn and Johnston, 1994).

Fig. 4. caf1 and ccr4 deletions are hypersensitive to CLB2 overexpression. (A) Yeast strains were grown on either YEP-glucose or YEP-galactose medium as indicated. ccr4Δ is strain CG378-1 (ccr4) and CCR4 is strain CG378 (wt). The presence of the YIpG7CLB2 plasmid integrated into the genome is as indicated. (B) Same as (A) except caf1 is strain 935-2-3 and CAF1 is strain 935-2.

Fig. 5. Expression of the CCR4 and CAF1 transcripts is not under cell cycle control. A culture of strain CG378 (wild-type) was synchronized by the α-factor method and samples were taken to determine the levels of each of the mRNA transcripts CCR4, CAF1, ACT1, and DBF2 by RNA blot analysis (Johnston et al., 1990). Samples were also taken to determine the percentage of cells with buds. Autoradiograms of the blot are shown, each of the bands corresponding to the time points indicated on the budding curve shown above. The results of the RNA blot analysis of mRNA samples taken from the log phase culture immediately prior to synchronization are shown in separate boxes on the left.
Discussion

DBF2 is a component of the CCR4 complex

DBF2, a cell cycle-regulated protein kinase, was identified by the two-hybrid assay as interacting with the general transcriptional regulator CCR4. The physical in vivo interaction of DBF2 with CCR4 was confirmed by co-immunoprecipitation studies. CAF1, which is tightly associated with CCR4, also interacted in the two-hybrid assay with DBF2 and co-immunoprecipitated with it. In addition, we demonstrated that DBF2 co-purified with CAF1 and CCR4 following two chromatographic steps, indicating that all three proteins are together in one complex. Whether CCR4 or CAF1 bind directly to DBF2 is not known. The CCR4 complex contains additional proteins besides CAF1 and DBF2 (Draper et al., 1994) and it may be that these proteins stabilize the complex between CCR4, CAF1 and DBF2. In support of this idea, the dual two-hybrid interactions between CCR4, CAF1 and DBF2 were not affected by the absence of the third factor, consistent with a large complex stabilized by multiple interactions between many proteins.

The physical association of DBF2 with CAF1 and CCR4 does not imply, however, that all of the DBF2 in the cell is found in the CCR4 transcriptional regulatory complex. The co-purification of LexA–DBF2 with CCR4 and CAF1-6His indicated that not all of the LexA–DBF2 was present in the CCR4 complex (Figure 2). While this can be interpreted to mean that a significant amount of DBF2 is not with CCR4, LexA–DBF2 was overexpressed relative to the amount of DBF2 in the cell and was expressed in a non-cell cycle-controlled manner. DBF2 has also been shown to act at least at two different points in the cell cycle (Johnston et al., 1990; Donovan et al., 1994), and it is possible that it associates with different proteins at different stages in the cell cycle.

DBF2 links the function of the CCR4 complex to cell cycle regulation

Mutations in DBF2 result in a dumb-bell shape cell morphology, with cells characterized by divided chromatin and fully extended spindles indicative of a block in late mitosis. The expression of the DBF2 gene, the phosphorylation state of the protein and DBF2 protein kinase activity are all under cell cycle control. The conversion of DBF2 from the phosphorylated to the non-phosphorylated form coincides with the point of action of DBF2 at the end of mitosis, suggesting that the non-phosphorylated DBF2 contains the protein kinase activity. Our observation that CCR4 immunoprecipitates were able to phosphorylate histone H1 in vitro indicates that this CCR4 complex contains a protein kinase. The fact that this kinase activity increased when the B42–DBF2 protein was present indicates that it is the DBF2 in the complex which is responsible for this kinase activity.

The physiological substrates of DBF2 remain, however, undefined. Neither CCR4 nor CAF1 were phosphorylated in vitro by DBF2, although DBF2 itself became phosphorylated in vitro. In addition, the association of DBF2 with CCR4 and with CAF1 did not require DBF2 kinase activity. Because defects in CCR4 and CAF1 resulted in a cell cycle delay in late mitosis in which increased numbers of cells had divided chromatin and elongated spindles, and because a caf1 or ccr4 disruption in combination with excess CLB2 protein resulted in a late mitotic block, it is clear that CCR4 and CAF1, in conjunction with DBF2, are important factors for progression through mitosis.

However, the CCR4 complex does not function solely to bind DBF2, since the phenotypes resulting from defects in these respective factors were overlapping but not identical. A dbf2 disruption was synthetically lethal in combination with swi5 (Toyn et al., 1996), dbf20 (Toyn et al., 1991) or sic1 (Donovan et al., 1994) whereas a ccr4 disruption did not display these phenotypes. Moreover, the cell cycle regulation of DBF2, CCR4 and CAF1 gene expression was distinct. While defects in these genes affected cell cycle progression, the ccr4 and caf1 phenotypes were less severe. This suggests that the essential DBF2 function in late mitosis is not shared by CCR4 and CAF1, although other functions in late mitosis are shared. Conceivably, DBF2 may have an adventitious role in the CCR4 transcriptional complex; that is to say it functions principally in pathways controlling the end of mitosis and secondarily in transcriptional events, and that these latter events are not essential for the telophase- G1 transition. Alternatively, as mentioned above, not all of the DBF2 in the cell may be complexed with CCR4 and CAF1, which would result in some DBF2 having partial and separate functions in the cell. In addition, the CCR4 complex may have multiple roles and contacts. For example, inactivating CCR4 in this complex would affect a subset of interactions and processes resulting in phenotypes that may be only partially similar to inactivating DBF2 or any other component of the complex. Such multi-faceted roles for different components of protein complexes are often observed as, for example, in the yeast transcriptional holoenzyme (Hengartner et al., 1995).

DBF2 affects transcription

While dbf2 mutations were identified originally as causing a block in cell cycle progression, a dbf2 deletion was viable and resulted in transcriptional phenotypes similar to those observed for ccr4 or caf1 disruptions. A dbf2 disruption was (i) staurosporine and caffeine sensitive, the former of which was suppressible by sorbitol, indicative of an effect on cell wall integrity genes; (ii) resulted in a 37°C glycerol defect; (iii) suppressed spt10-enhanced ADH2 expression; and (iv) decreased the ability of some but not all LexA transactivators to function. DBF2 has also been found to affect the expression of several lacZ reporter plasmids (unpublished observations). These effects suggest that DBF2 functions as a transcriptional regulator.

One model describing DBF2 control of cell cycle events would be that DBF2 phosphorylates factors which allow specific cell cycle genes to be transcribed during late mitosis. DBF2 and the CCR4 complex might represent, therefore, a signaling processor. Different signals generated, for example, from a shift to non-fermentative growth conditions or from progression through the cell cycle would influence the activity and interactions of components of the CCR4 complex and result in bringing about a subset of specific transcriptional events. Since CCR4 acts at a post-chromatid remodeling event in its effects on transcription (M.Caserta, personal communication), DBF2 would also presumably act at this level. The CCR4
**Materials and methods**

**Yeast strains, growth conditions and enzyme assays**

Yeast strains used in this study are listed in Table V. Yeast generally were cultured on YEP medium (1% bactopectone, 1% yeast extract) containing either 8% glucose or 3% ethanol or on minimal medium lacking uracil, histidine and/or tryptophan containing 8% glucose, 2% each of ethanol and glycerol, or 2% each of raffinose and galactose. ADH II enzyme assays were conducted as previously described following growth on YEP medium, and β-galactosidase assays were conducted on minimal medium as previously described (Cook et al., 1994). α-Factor arrest was conducted as previously described (Johnston et al., 1990).

**Two-hybrid screen**

A yeast interaction library containing yeast genomic sequences fused to the B42 activation domain (Zverov et al., 1993) was used to transform (Bo et al., 1983) strain EGY188 containing the LexA(1–87)-CCR4 fusion and the LexA4–lacZ reporter 34 that has eight LexA-binding sites upstream of the GAL4–lacZ reporter (Cook et al., 1994). Identification of colonies and screening for galactose and plasmid dependence were done as described (Zverov et al., 1993; Draper et al., 1995).

**Construction of fusion proteins**

The B42–DBF2 full-length fusion was a gift of S Komarnitsky and was constructed by cloning a 2 kb HinclI fragment of pRS304–DBF2-cmyc (Toyn and Johnston, 1994) into the LexA202-1 vector whose BamHI site was filled in with Klenow. To construct CAF1–6His, two primers, 5’ ACTCA-GAATTCCGGCCG-3’ and 5’ CCCCCCAGAGCTGGCTCCTACATTACGCG3’ were used to amplify the CAF1 gene and to create a HindIII site at the end of the CAF1 coding sequence. The PCR-amplified product was digested with ClaI and HindIII and the isolated ClaI–HindIII DNA fragment was cloned into the ClaI–HindIII sites of pMD120 (contains full-length CAF1 fused to LexA-202); the resulting plasmid was designated pMLF1. The 1.4 kb BamHI–HindIII fragment of pMLF2 was then used to replace the BamHI–HindIII segment of pHISC, a SP72 cloning vector containing a 6His oligonucleotide. The newly constructed plasmid, designated pMLF2, contains the intact coding sequence of CAF1–6His. The LexA–CAF1–6His fusion was made by inserting the 1.4 kb BamHI–SalI fragment of pMLF2 into the BamHI–SalI sites of LexA-202. These LexA–CAF1 derivatives were designated pMLF3 for the 87 version and pMLF4 for the 202 version. Finally, the CAF1–6His integration plasmid, designated pMLF5, was constructed by replacing the PstI–SalI fragment of pMD103 with the 960 bp PstI–SalI fragment of pMLF2.

**Immunoprecipitation and kinase activity assay**

Yeast strain EGY188 containing the plasmid B42–DBF2 was grown on minimal medium lacking tryptophan and containing either 2% each of galactose and raffinose or 2% glucose. Cells were pelleted and the whole cell protein was extracted in lysis buffer (50 mM K phosphate, pH 7.7, 150 mM KCl, 20% glycerol, 1 mM NaPP, 1 mM NaF, 1 mM EDTA, 2 mM MgCl$_2$, 1% NP-40 plus protease inhibitors). Then 250 μg of protein was incubated with 2 μg of CCR4 antibody for 45 min at 4°C and mixed with 20 μl of protein A–agarose for an additional 30 min. The beads were pelleted by centrifugation in a microcentrifuge and washed in 3×1 ml of the lysis buffer, 1 ml of the 87 buffer with 1 M KC1 and 1 ml of the Triton X-100 wash buffer. SDS sample buffer (20 μl) was added to the beads and the beads were boiled for 4 min before being loaded on a 10% SDS–PAGE gel. Western blot analysis was carried out as described (Draper et al., 1995). After the immunoprecipitations, in vitro kinase assays using H1 histone were conducted as described by Toyn and Johnston (1994). Immunoprecipitations using anti-CCR4 or anti-CAF1 antibody were conducted as described previously (Draper et al., 1994, 1995), and ECL analysis (Pierce) was conducted according to the manufacturer’s instructions. Reimmunoprecipitation experiments were carried out exactly as described in Vallari et al. (1992).
medium lacking histidine and tryptophan and containing 2% glucose. Cells were pelleted by centrifugation at 3000 r.p.m. at 4°C for 5 min. The pelleted cells were washed twice in buffer A. The whole cell protein was extracted in 0.5% buffer A containing 20% glycerol with protease inhibitors (0.5 ml/1 g wet cells) in a bead-beater device by 6×30 s blasts. The lysate was cleared by a 10 min spin in a microcentrifuge and the supernatant was then centrifuged at 64 000 r.p.m. for 90 min. The lipid was carefully removed and the clear supernatant was pooled. Imidazole was added to the extract to a final concentration of 25 mM, and the extract was then mixed with 0.5 ml of pre-equilibrated Ni²⁺–NTA–agarose beads for 60 min with end-to-end rocking in the cold room. The mixture was loaded onto a 5 ml Bioc-Rad column and the beads were washed in 10 ml of buffer B (buffer A containing 500 mM KOAc and 25 mM imidazole). The bound proteins were eluted in 2 ml of buffer C (buffer A containing 250 mM imidazole). The Ni²⁺-eluant was loaded directly on a 1 ml FPLC Mono-Q column, the bound protein was eluted with a 20 ml linear salt gradient of buffer E (buffer A containing 1 mM EDTA and 1 mM dithiothreitol) to buffer F (buffer E containing 2 M KOAc) and 1 ml fractions were collected.

Test of sensitivity of yeast strains to CLB2 overexpression

The integrating vector YIpG7CLB2 (Shirayama et al., 1994) was linearized by digestion with the restriction enzyme Stul and introduced into yeast. Integration by homologous recombination of the plasmid into the UR13 locus and plasmid copy number were confirmed by Southern analysis. Briefly, yeast genomic DNA was digested with the restriction enzyme EcoRI. A Southern blot of this DNA was probed with a radiolabeled CLB2 DNA fragment. The endogenous CLB2 locus produced a DNA fragment of 5 kb, and the integrated plasmid-borne CLB2 produced a DNA fragment of ~10 kb. When the two integrated copies of the plasmid were present, a third DNA fragment of ~8 kb (the size of the YIpG7CLB2 plasmid) could be detected. Mutant strains and isogenic control strains containing a defined plasmid copy number were then tested for growth on YEP galactose agar medium. We have found that different (non-mutant) strains respond very differently to the presence of a single copy of YIpG7CLB2 on galactose medium. Some strains are unable to grow on YEP galactose in the presence of YIpG7CLB2, whereas others are able to grow only when two copies of the plasmid are present. Still other strains (e.g. CG378) are able to grow on galactose even in the presence of two copies of the plasmid. Whether this reflects differences in the level of galactose-induced transcription or differences in the sensitivity of the B cyclin kinase to CLB2 overexpression, we do not know. For the experiments described in this study, one copy of the YIpG7CLB2 plasmid was found to arrest growth of caf1 in the 935-2 genetic background, whereas two copies were required to arrest growth of ccr4 in the CG378 genetic background.

RNA blot analysis

Total RNA was extracted from yeast as previously described (Toyn and Johnston, 1994); 5 μg samples were denatured with glyoxal, separated by agarose gel electrophoresis and transferred to a GeneScreen hybridization membrane (DuPont NEN Research Products, Boston, MA). Radiolabeled DNA probes for DNA–RNA hybridization were prepared using random oligonucleotide priming (Multiprime DNA labelling kit, Amersham). NTA–agarose beads for 60 min with end-to-end rocking in the cold room. The mixture was loaded onto a 5 ml Bio-Rad column and the beads were washed in 10 ml of buffer B (buffer A containing 500 mM KOAc and 25 mM imidazole). The bound proteins were eluted in 2 ml of buffer C (buffer A containing 250 mM imidazole). The Ni²⁺-eluant was loaded directly on a 1 ml FPLC Mono-Q column, the bound protein was eluted with a 20 ml linear salt gradient of buffer E (buffer A containing 1 mM EDTA and 1 mM dithiothreitol) to buffer F (buffer E containing 2 M KOAc) and 1 ml fractions were collected.

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