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 Diceshofer, 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* (Sakai *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 (Johnston *et al.*, 1990). Consistent with a mitotic role for DBF2, the gene is expressed under cell cycle control in M phase. *DBF2* encodes a protein kinase and this activity is also cell cycle regulated, with a peak in late mitosis (Toyn and Johnston, 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 *GALI* promoter (Zervos *et al.*, 1993). Fifty six colonies that displayed galactose-

Table I. Two-hybrid interactions of DBF2 with CCR4 and CAF1

LexA fusion	B42 fusion	β -Gal (U/mg)
LexA-CCR4	B42	30
LexA-CCR4	B42-DBF2 (205–561)	150
LexA-CCR4	B42-DBF2 (1–561)	170
LexA-CCR4	B42-DBF2 (K195T)	110
LexA	B42-DBF2 (205–561)	1.3
LexA-CCR4 (Δ LRR)	B42-DBF2 (205–561)	<1
LexA-CAF1	B42	110
LexA-CAF1	B42-DBF2 (205–561)	210
LexA-CAF1	B42-DBF2 (1–561)	1700
LexA-CAF1	B42-DBF2 (K195T)	1500

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* (Cook *et al.*, 1994). 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). B42-DBF2 (K195T) is the same as B42-DBF2 (1–561) except for a threonine substitution at residue 195.

dependent activation of both the *LexA_{op}-LEU2* and the *LexA_{op}-lacZ* reporters were isolated from $\sim 2 \times 10^6$ transformants. Library plasmids were isolated from the 56 colonies, and each was found to contain one of seven genes, two of which were identified previously. One of these, *CAF1*, encodes a protein which has been shown previously to interact with LexA-CCR4 and to be physically associated with CCR4 *in vivo* (Draper *et al.*, 1995). The second gene, designated *CAF2*, was found to be identical to the yeast gene, *DBF2*. DBF2 is a cell cycle-regulated protein kinase that plays an important role in the telophase to G₁ transition (Toyn and Johnston, 1994).

As summarized in Table I, the B42-DBF2 fusion containing DBF2 residues 205 to its C-terminus interacted with LexA-CCR4 but failed to interact with the LexA moiety alone. DBF2 (205–561) is truncated midway in its protein kinase domain and would be expected to be inactive as a protein kinase. To confirm the interaction between CCR4 and DBF2, we constructed a full-length DBF2 fused to B42 and expressed it along with LexA-CCR4. The B42-DBF2 (full-length) fusion, which is capable of complementing phenotypes associated with a *dbf2* disruption (data not shown), also interacted with LexA-CCR4 and did so to the same extent as the truncated B42-DBF2 fusion (Table I). A LexA-CCR4 derivative containing a deletion in the LRR region (Draper *et al.*, 1994) failed to interact with DBF2 (Table I), suggesting that the interaction observed between CCR4 and DBF2 was dependent on the presence of the LRR region in the CCR4 protein.

We also analyzed the ability of B42-DBF2 to interact with LexA-CAF1. The full-length B42-DBF2 fusion displayed an interaction with either LexA-CAF1 (residues 124–441) (Table I) or with LexA-CAF1 (full-length) (data not shown). In contrast, truncated B42-DBF2 (205–561) did not interact with either LexA-CAF1 construct (Table I; data not shown). Also, the interactions between CCR4 and DBF2, CCR4 and CAF1, and CAF1 and DBF2 essentially were unaffected by deletion of the gene encoding the other factor, CAF1, DBF2 and CCR4, respectively

Fig. 1. Co-immunoprecipitation of B42-DBF2 with CCR4 and CAF1.

(A) Extracts from strain EGY188 containing either B42-DBF2 or B42-SIP1 were incubated with either anti-CCR4 or anti-LexA antibodies and the resulting immunoprecipitates 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 (*ccr4*); lane 2, crude extract from EGY188-c1 (*caf1*); 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-c1 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).

(data not shown). Other factors, therefore, appear to mediate or stabilize the association of these three proteins.

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 or HA1 (Figure 1). The B42-DBF2 protein was co-immunoprecipitated specifically with the CCR4 antibody (Figure 1A, lane 4) but was not immunoprecipitated by an antibody raised against the LexA protein (lane 5). In addition, B42-DBF2 was found not to be immunoprecipitated from extracts prepared from a strain lacking CCR4 protein (Figure 1B, compare lanes 4 and 5). In a control experiment, a B42-SIP1 fusion protein [SIP1 is a protein associated with the SNF1 protein kinase (Yang *et al.*, 1992) and has been shown not to be associated with the CCR4 complex, data not shown] did not co-immunoprecipitate with CCR4 (Figure 1A, lane 3). These data indicate

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: Ni^{2+} -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 that 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 Ni^{2+} -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 Ni^{2+} -NTA and Mono-Q columns (Figure 2B). In a control experiment, LexA alone was not retained on the Ni^{2+} -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

Fig. 2. Co-purification of Lex–DBF2 with CAF1 and CCR4. (A) Ten ml of extracts from strain MLF6-3 (*CAF1-6His*) were bound to Ni^{2+} -NTA-agarose beads and, after imidazole elution, the 3 ml of eluent were subjected to Mono-Q chromatography. The resultant 1 ml chromatographic fractions (5 ml for flowthrough) were subjected to Western analysis following 10% SDS-PAGE. CCR4 and CAF1-6His proteins were detected as indicated with anti-CCR4 and anti-CAF1 antibodies. It should be noted that with this anti-CAF1 antibody, CAF1 protein cannot be detected in crude extracts. Also, in crude extracts, the CCR4 antibody recognizes the non-specific protein that is larger than CCR4. CrEx, 15 μl of crude extract; Ni-eluant, 50 μl of Ni^{2+} -NTA-agarose eluant with imidazole; flow-through, 20 μl of flowthrough of Ni^{2+} eluant subjected to Mono-Q chromatography; Mono-Q fractions, 20 μl of fractions obtained by salt elution of Ni^{2+} eluant proteins subjected to Mono-Q chromatography. (B) Yeast extracts were prepared from MLF6 (*CAF1-6His*) containing the LexA–DBF2 plasmid. Lane designations are the same as in (A), except that 30 μl of the different samples were analyzed. The Western was developed with antibody directed against CAF1 and LexA. We estimate that ~30% of the LexA–DBF2 bound to the Ni^{2+} -NTA-agarose column co-migrated with CAF1-6His following Mono-Q chromatography. (C) Extracts were prepared from strain MLF6 containing LexA-202-3 (LexA) or LexA–DBF2. Lane designations are the same as in (A). The Western was developed with antibody specific to LexA.

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.

immunoprecipitates (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 subsequently 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 ADH II 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 *ADR1-5^c* allele which displays high ADH II enzyme levels under repressed conditions similar to those observed in an *spt10*-containing strain. In contrast, a deletion of *DBF20*, a non-cell cycle-controlled homolog of *DBF2*, had no effect on *spt10*-enhanced *ADH2* expression (Table II).

ccr4 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 glycerol 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 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

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 were 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-CCR4-1-345 by ~2- to 3-fold, although it had little or less effect on LexA-CAF1 or LexA-ADR1-full length activation (data not shown). These results are similar to those observed for *caf1* and *ccr4* defects, but the effects are not as general as for the defects in the *CAF1* and *CCR4* genes. The above results indicate that DBF2 is required for processes similar to those that require CCR4 and CAF1. More importantly, they indicate that DBF2 affects several different transcriptional processes and behaves as a functional component of the CCR4 complex.

ccr4 and *caf1* strains have cell cycle-related phenotypes

The finding that DBF2 is physically associated with both CCR4 and CAF1 suggested that these proteins cooperate in cell regulation, and would therefore have related phenotypes when mutated. Because DBF2 functions at the end of mitosis (Johnston *et al.*, 1990; Toyn and Johnston, 1994), we investigated whether deletion of CCR4 or CAF1 caused similar cell cycle defects. Two different tests indicated that *ccr4* and *caf1* deletion strains were partially defective for cell cycle progression at the end of mitosis: firstly, they displayed an increased proportion of late

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

	ADH II (mU/mg)
<i>spt10</i> ^a	87
<i>dbf2spt10</i> ^a	9.0
<i>ADR1-5^c</i>	100
<i>dbf2 ADR1-5^c</i>	120
<i>spt10</i> ^c	210
<i>dbf20 spt10</i> ^c	200
<i>spt10</i> ^d	175
<i>ccr4 spt10</i> ^d	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.

^aSegregants from crosses 994-2×991-2-6d and 1013-2a×1013-6a.

^bSegregants from cross 787-6b×1007-1-4a.

^cSegregants from cross 808-5c×1044-5a.

^dData from Denis (1984).

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

Strain	Relevant genotype	12°C	12°C + sorb.	37°C	37°C + sorb.	Gly 37°C	Stauro	Stauro + sorb.	Caffeine	LiCl
188	wt	+	+	+	+	+	+	+	+	+
188-1	<i>ccr4</i>	–	+	w	+	–	–	+	–	–
188-c1	<i>caf1</i>	–	w	–	+	–	–	+	–	w
S7-4A	<i>dbf2</i>	w	+	w	+	–	–	+	–	–

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

Yeast strains	Buds (%)	Divided chromatin (%)	Budded cells with divided chromatin (%)
wt ^a	62	15	24
<i>ccr4</i> ^b	75	44	57
wt ^c	45	10	22
<i>caf1</i> ^d	54	20	37

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).

^awt, strain 612-1d; ^b*ccr4*, strain 612-1d-2A; ^cwt, strain 935-2; ^d*caf1*, strain 935-2-3.

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 YlpG7CLB2 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.

mitotic cells in log phase cultures, and, secondly, they were hypersensitive to *CLB2* overexpression.

Log phase *ccr4*, *caf1* and isogenic control cultures were stained with 4',6'-diamidino-2-phenylindole (DAPI), and the percentage of cells with buds and with divided chromatin was counted, as an indicator of the number of telophase cells (Table IV). Both mutants showed an increase in the budded population, consistent with a mitotic delay, but, more importantly, the *ccr4* and *caf1* cultures had ~3-fold and 2-fold increases in the percentage of total cells with divided chromatin, respectively. This suggests that the *ccr4* and *caf1* genotypes result in a delayed exit from mitosis, at least in terms of causing some of the cells to spend more time in telophase.

Deactivation of the B cyclin kinase activity is necessary for the exit from mitosis, and it has been shown that overexpression of *CLB2*, or expression of non-degradable

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.

CLB2, prevents B cyclin kinase deactivation, causing a stage-specific arrest of the cell cycle in telophase (Surana *et al.*, 1993). Furthermore, yeast mutants that are partially defective for B cyclin kinase deactivation, as a result of mutations in the relevant regulatory genes, are hypersensitive to overexpression of *CLB2* (Shirayama *et al.*, 1994; Toyn *et al.*, 1997). To test for involvement in B cyclin kinase deactivation, the *GAL7-CLB2* overexpression integrating plasmid (Shirayama *et al.*, 1994) was integrated into *ccr4*, *caf1* and isogenic control strains, and growth was tested on galactose medium (Figure 4). Both *ccr4* and *caf1* strains were unable to grow on galactose medium when the *GAL7-CLB2* gene was expressed, whereas isogenic control strains were not affected. These results confirmed that both *CCR4* and *CAF1* proteins contribute to the deactivation of the B cyclin kinase *in vivo*. Thus, the three presently identified components of the *CCR4* transcription complex, i.e. *CCR4*, *CAF1* and *DBF2*, all affect mitotic exit. It therefore seems likely that some of the target genes regulated by this transcription complex are involved in the regulation of mitotic exit.

Other phenotypes typically observed with a *dbf2* disruption were not observed, however, with *ccr4* or *caf1* defects. A *dbf2* disruption is synthetically lethal in combination with *dbf20* (Toyn *et al.*, 1991), *sic1* (Donovan *et al.*, 1994) or *swi5* (Toyn *et al.*, 1997). Neither *ccr4* nor *caf1* were synthetically lethal with these mutated alleles (data now shown). Moreover, while *DBF2* expression is cell cycle-regulated, reaching a maximum at the anaphase to telophase transition (Figure 5; Johnston *et al.*, 1990; Toyn and Johnston, 1994), neither *CCR4* nor *CAF1* gene expression was found to be under cell cycle control (Figure 5).

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 transcription 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-chromatin remodeling event in its effects on transcription (M.Caserta, personal communication), DBF2 would also presumably act at this level. The CCR4

Table V. Yeast strains

Strain	Relevant genotype
EGY188	<i>MATa ura3 his3 trp1 leu2 LexA-LEU2</i>
EGY188-c1	isogenic to EGY188 except <i>caf1::URA3</i>
EGY188-c1-1	isogenic to EGY188-c1 except <i>caf1::ura3</i>
EGY188-1	isogenic to 188 except <i>ccr4::URA3</i>
612-1d	<i>MATa ura3 his3 trp1 leu2 adh1-11</i>
612-1d-2A	isogenic to 612-1d except <i>ccr4::HIS3</i>
991-1-1b	<i>MATα ura3 his3 trp1 leu2 dbf2::URA3</i>
991-1-1b-3	isogenic to 991-1-1b except <i>trp1::cmv-DBF2-TRP1</i>
1013-2a	<i>MATa adh1-11 ura3 trp1 leu2 spt10::TRP1</i>
1013-6a	<i>MATα adh1-11 ura3 trp1 leu2 his3 spt10::TRP1 dbf2::LEU2</i>
991-2-6d	<i>MATα adh1-11 trp1 ura3 leu2 dbf2::LEU2</i>
994-2	<i>MATα adh1-11 trp1 ura3 his3 leu2 spt10::TRP1</i>
1007-1-4c	<i>MATα adh1-11 ura3 trp1 his3 leu2 dbf2::URA3 caf1::LEU2</i>
787-6b	<i>MATα adh1-11 adr1-1::TRP1-ADR1-5^c ura3 his3 leu2 trp1</i>
808-5c	<i>MATα adh1-11 ura3 trp1 leu2 his3 spt10::LEU2</i>
1044-5a	<i>MATα adh1-11 ura3 trp1 leu2 his3 dbf20::TRP1</i>
935-2	<i>MATα adh1-11 his3 leu2 ura3</i>
935-2-3	isogenic to 935-2 except <i>caf1::LEU2</i>
S7-4A	<i>MATa dbf2::URA3 ura3 leu2 ade5 trp1 his7</i>
S7-4A-c-myc	isogenic to S7-4A except <i>trp1::cmv-DBF2-TRP1</i>
CG378	<i>MATa ura3 leu2 trp1</i>
CG378-1	isogenic to CG378 except <i>ccr4::URA3</i>
EGY191-2	<i>MATα ura3 his3 trp1 LexA-LEU2 caf1::LEU2</i>
MLF6	isogenic to EGY191-2 except <i>trp1::CAF1-6His-TRP1</i>

complex might stabilize or recruit the holoenzyme complex (Hengartner *et al.*, 1995) or aid transcriptional activators in recruiting the transcriptional machinery. DBF2, under cell cycle control, would be one part of the CCR4 complex that aids in turning on cell cycle-related genes. CCR4, on the other hand, which is capable of activating transcription in a carbon source-dependent manner when bound to DNA (Draper *et al.*, 1994), may also play a role in effecting the transcription of non-fermentative genes. This mediating or signaling role of CCR4 and DBF2 function would place these factors as intermediates between signal transduction pathways and the final transcriptionally competent initiation complex.

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 (Zervos *et al.*, 1993) was used to transform (Ito *et al.*, 1983) strain EGY188 containing the LexA(1–87)–CCR4 fusion and the *LexA_{opp}-lacZ* reporter 34 that has eight LexA-binding sites upstream of the *GAL1-lacZ* reporter (Cook *et al.*, 1994). Identification of colonies and screening for galactose and plasmid dependence were done as described (Zervos *et al.*, 1993; Draper *et al.*, 1995).

Construction of fusion proteins

The B42–DBF2 full-length fusion was a gift of S.Komarnitsky and contained the complete sequence of DBF2 fused to B42 in the pJG4-5 vector (Zervos *et al.*, 1993). The B42–DBF2-K195T fusion was constructed by placing a 2.3 kb *SalI* fragment of DBF2-K195T at the *XhoI* site of the pJG4-5 vector. The LexA–DBF2 fusion was constructed by

cloning a 2 kb *HincII* fragment of pRS304-DBF2-cmyc (Toyn and Johnston, 1994) into the LexA202-1 vector whose *BamHI* site had been filled in by Klenow. To construct *CAF1-6His*, two primers, 5'ACTCA-GAAAATCAGGC3' and 5'CCCCCAAGCTTGGTCCCATCAATA-CCG3' were used to amplify the *CAF1* gene and to create a *HindIII* site at the 3' 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-202-1. 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 overnight 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_i, 1 mM NaF, 1 mM EDTA, 2 mM MgCl₂, 1% NP-40 plus protease inhibitors). Then 250 μ g of protein was incubated with 2 μ g of CCR4 antibody for 45 min at 4°C and mixed with 20 μ l of protein A-agarose for an additional 30 min. The beads were pelleted by centrifugation in a microcentrifuge and washed in 3 $\times$ 1 ml of the lysis buffer, 1 ml of lysis buffer with 1 M KCl and 1 ml of the Triton X-100 wash buffer. SDS sample buffer (20 μ l) 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. Re-immunoprecipitation experiments were carried out exactly as described in Vallari *et al.* (1992).

Chromatographic analysis of the CCR4 complex

The yeast strain MLF6 (EGY191-2 except *trp1::CAF1-6His-TRP1*) containing the LexA–DBF2 plasmid was grown overnight on minimal

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 3× buffer A (20 mM Tris-HCl, pH 7.9, 150 mM KOAc, 1 mM MgOAc 1 mM EDTA, 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 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.

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 *Stu*I and introduced into yeast. Integration by homologous recombination of the plasmid into the *URA3* locus and plasmid copy number were confirmed by Southern analysis. Briefly, yeast genomic DNA was digested with the restriction enzyme *Eco*RI, and 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 unable 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 International plc, Amersham, UK), and corresponded to coding regions of the genes concerned. For *CCR4*, a 2 kb *Bam*HI-*Eco*RI genomic DNA fragment was used, and for *CAF1* a 0.5 kb *Eco*RI-*Eco*RI fragment from the *LexA-CAF1* fusion vector was used. Hybridization of probes to the blot was detected by autoradiography and phosphorimage analysis.

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