# Dhh1p, a Putative RNA Helicase, Associates with the General Transcription Factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*

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ABSTRACT

The POP2 (Caf1) protein in *Saccharomyces cerevisiae* affects a variety of transcriptional processes and is a component of the Ccr4p complex. We have isolated five multicopy suppressor genes of a *pop2* deletion mutation: *CCR4*, *DHH1* (a putative RNA helicase), *PKC1*, *STM1*, and *MPT5* (multicopy suppressor of pop two). Overexpression of either the *CCR4* or *DHH1* genes effectively suppressed phenotypes associated with *pop2* mutant cells; overexpression of *PKC1*, *STM1*, or *MPT5* genes produced only partial suppression. Disruption of the *CCR4* or *DHH1* genes resulted in phenotypes similar to those observed for *pop2* cells. In addition, overexpression of the *DHH1* gene also suppressed the *ccr4* mutation, suggesting a close relationship between the *POP2*, *CCR4*, and *DHH1* genes. Two-hybrid analysis and coimmunoprecipitation experiments revealed that Pop2p and Dhh1p interact physically, and these and other data suggest that Dhh1p is also a component of the Ccr4p complex. Finally, we investigated the genetic interaction between factors associated with *POP2* and the *PKC1* pathway. The temperature-sensitive growth defect of *dhh1* or *mpt5* cells was suppressed by overexpression of *PKC1*, and the defect of *mpk1* cells was suppressed by overexpression of *MPT5*. These results and phenotypic analysis of double mutants from the *POP2* and *PKC1* pathways suggested that the *POP2* and the *PKC1* pathways are independent but have some overlapping functions.

THE *POP2* (*CAF1*) gene is required for glucose derepression of gene expression in *Saccharomyces cerevisiae* (Sakai *et al.* 1992; Draper *et al.* 1995). The *pop2* mutant cells exhibit many defects, including reduced levels of reserve carbohydrates, resistance to glucose derepression, temperature sensitivity for growth, increased *PGK1* transcription during stationary phase, and reduced levels of alcohol dehydrogenase II, isocitrate lyase, and invertase (Sakai *et al.* 1992; Draper *et al.* 1995). Pop2p has been shown to be part of the Ccr4p complex (Draper *et al.* 1995; Liu *et al.* 1997). Ccr4p, a general transcription factor, is required for the transcription of many genes, including glucose-repressible *ADH2*, and the pleiotropic nature of defects in *CCR4* is similar to that in *POP2* (Denis and Malvar 1990; Malvar *et al.* 1992; Sakai *et al.* 1992). Homologs of the *POP2* gene have been identified from humans, mice, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (Draper *et al.* 1995). The high degree of evolutionary conservation and their functional interchangeability suggest that the *POP2* gene plays an important functional role in cells.

We have identified several new phenotypes of *pop2* mutants. First, *pop2* cells are sensitive to staurosporine, a potent inhibitor of protein kinase C that is encoded by *PKC1*. Second, *pop2* cells are also sensitive to caffeine (Liu *et al.* 1997). In addition, the temperature-sensitive phenotype of *pop2* is suppressed by the addition of 1 M sorbitol to the medium. These phenotypes are characteristic of mutants involved in the *PKC1-MPK1* pathway (Thevelein 1994), which controls cell wall integrity. Cells carrying *pkc1*Δ or *mpk1*Δ cannot use glycerol as a sole carbon source (Costigan *et al.* 1994), and this suggests the involvement of the *PKC1-MPK1* pathway in the transcription of genes required for glycerol utilization. To understand the functions of Pop2p, we have searched for multicopy suppressor genes of a *pop2* deletion mutation. This approach has proved to be quite useful for analyzing many cellular processes such as the *SNF1* kinase pathway (Estruch and Carlson 1993; Hubbard *et al.* 1994) and the *PKC1-MPK1* protein kinase pathway (Lee *et al.* 1993a; Nickas and Yaffe 1996). Using this approach, we hoped to obtain genes encoding proteins with functional relationships to *POP2*: negative regulators of an antagonistic pathway or positive regulators of

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**TABLE 1**

**List of yeast strains**

| Strain | Genotype | Source |
|---|---|---|
| D40 | *MAT***a***/MATα ade8/ade8 aro7/ARO7 his3/his3 leu2/leu2 TRP1/trp1 ura3/ura3* | This work |
| A475 | *MAT***a** *can1 gal2 leu2 met14 trp1 ura3 rgr1Δ2::URA3* | Sakai, 1990 |
| A880 | *MAT***a** *aro7 his3 leu2 ura3 pop2-Δ3::URA3* | This work |
| A1123 | *MATα leu2 met14 trp1 ura3* | This work |
| A1152 | *MATα aro7 his3 leu2 tmet14 trp1 ura3 stt1-1 (pkc1$^{ts}$)* | This work |
| A1158 | *MAT***a** *aro7 his3 leu2 trp1 ura3 pkc1Δ::HIS3* | This work |
| A1385 | *MATα ade8 his3 leu2 trp1 ura3 dhh1Δ::URA3* | This work |
| A1413 | *MAT***a** *ade8 his3 leu2 trp1 ura3 mpt5Δ::HIS3* | This work |
| A1454 | *MAT***a** *aro7 ade8 his3 leu2 ura3 stm1Δ::ADE8* | This work |
| A1522 | *MATα aro7 ade8 his3 leu2 trp1 ura3 ccr4Δ::HIS3* | This work |
| 612-1d | *MAT***a** *adh1-11his3 leu2 trp1 ura3* | This work |
| 612-1d-d1 | *MAT***a** *adh1-11 his3 trp1 leu2 ura3 dhh1Δ::URA3* | This work |
| EGY188 | *MAT***a** *his3 leu2 trp1 ura3 LexAop-LEU2* | This work |
| EGY191 | *MATα his3 leu2 trp1 ura3 LexAop-LEU2* | This work |
| L40 | *MAT***a** *his3 leu2 trp1 ura3 LYS::LexAop-HIS3 URA3::LexAop-lacZ* | T. Tanaka, Shimane University |
| DL251 | *MAT***a***/MATα can1/can1 his4/his4 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-52/ura3-52 bck1Δ::URA3/bck1Δ::URA3* | D. L. Levin, Johns Hopkins University |
| DL456 | *MAT***a***/MATα can1/can1 his4/his4 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-52/ura3-52 mpk1Δ::TRP1-/mpk1Δ::TRP1* | D. L. Levin |
| KAN128 | *MAT***a** *can1 his4 leu2-3,112 trp1-1 ura3-52 mkk1Δ::LUE2 mkk2Δ::URA3* | K. Irie, Nagoya University |

a downstream pathway. We report here the isolation of five multicopy suppressor genes of the *pop2* deletion mutation in *S. cerevisiae*. We also present genetic and biochemical evidence indicating that Pop2p, Ccr4p, and Dhh1p are part of the same complex of proteins. A possible interaction between the *POP2* and *PKC1-MPK1* pathways is discussed.

## MATERIALS AND METHODS

**Strains and genetic methods:** The strains of *S. cerevisiae* used in this study are listed in Table 1. Crossing, sporulation, and tetrad analyses were carried out by standard genetic methods (Sherman *et al.* 1986). Unless otherwise specified, the permissive and restrictive temperatures were 24° and 36°, respectively. The transformation of yeast was performed by the LiOAc method (Ito *et al.* 1983). *Escherichia coli* strains HB101 and JM109 were used as hosts for constructing and propagating plasmids. The transformation of *E. coli* was performed as described (Hanahan 1983). The basic culture medium used for *S. cerevisiae* was YPD medium containing 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose (Sherman *et al.* 1986). The synthetic medium was CSM medium containing 0.67% yeast nitrogen base without amino acids, 2% dextrose, and amino acids as required (Sherman *et al.* 1986). The media were solidified with 2% Bacto-agar for plates. To test the sensitivity of various mutants to staurosporine (Kyowa Medix, Tokyo, Japan), the drug was dissolved in dimethyl sulfoxide and added to YPD media to a final concentration of up to 5 μg/ml (Tamaoki *et al.* 1986; Yoshida *et al.* 1992). Tetrad dissection of heterozygous diploids carrying *pkc1Δ* was performed on a YPD plate containing 1 M sorbitol. Luria broth was supplemented with ampicillin for selection of the *E. coli* transformants as described (Maniatis *et al.* 1982).

**Preparation of DNA and RNA:** Preparation of *E. coli* DNA, Southern hybridization, and Northern hybridization were performed as described (Maniatis *et al.* 1982). The preparation of yeast DNAs and Northern analysis were performed as described (Sherman *et al.* 1986).

**ADH II assay:** Yeast cells were grown in YEP medium supplemented with either 8% glucose or 3% ethanol, and the activity of ADH II was measured as described previously (Cook *et al.* 1994).

**Isolation of multicopy suppressors of *pop2-Δ4*:** A yeast genomic DNA library (in YEp13, *LEU2* marker) was transformed to an A880 strain carrying the *pop2-Δ4* mutation (Sakai *et al.* 1992), and transformants were selected on CSM lacking leucine at 24° for 5 days. To isolate suppressors for the temperature-sensitive phenotype of *pop2* cells, transformants were replica plated on YPD, and the plates were further incubated at 36°. To isolate suppressors for the staurosporine-sensitive phenotype of *pop2* cells, the transformants were replica plated on YPD containing 1 μg/ml staurosporine. Of 25,000 transformants, 85 colonies were selected. The 85 suppressor plasmids were recovered, analyzed by Southern hybridization and restriction mapping, and divided into six classes. The largest class (containing 65 plasmids) was identified as *POP2* itself. The remaining five classes were tentatively called MPTs (multicopy suppressor of pop two). The classes of *MPT1*, *MPT2*, *MPT3*, *MPT4*, and *MPT5* contained 13, 3, 2, 1, and 1 plasmids, respectively. To localize the suppressor activities for these plasmids, individual restriction fragments were subcloned into the YEp213 vector and tested for their abilities to grow on YPD plates at the restriction temperature or YPD plates containing staurosporine. These *MPT* genes were further subcloned into pUC18, and the nucleotide sequences were determined by the Sanger method using a Sequenase Kit (United States Biochemical, Cleveland, OH). Nucleotide sequence analysis revealed that three genes had been identified previously. *MPT1* is *DHH1*, which encodes a putative RNA helicase (Strahl-Bolsinger and Tanner 1993), *MPT2* is identical to *CCR4*, which is required for the full expression of

the glucose-repressible *ADH2* gene (Denis and Malvar 1990; Malvar *et al.* 1992), and *MPT3* turned out to be *PKC1*, a yeast homolog of mammalian protein kinase C (Levin *et al.* 1990). *MPT4* is *STM1*, a guanine quartet–binding protein (Frantz and Gilbert 1995). *MPT5* is identical to *HTR1*. The name *HTR1* had been used for a different gene (Özcan *et al.* 1993); we refer to this gene as *MPT5*.

**Disruption of *DHH1, CCR4, PKC1, MPT4,* and *MPT5*:** The *dhh1Δ::URA3*, *ccr4Δ::HIS3*, *pkc1Δ::HIS3*, *stm1Δ::ADE8*, and *mpt5Δ::HIS3* alleles were constructed by the standard method and transformed into diploid (D40) cells to replace the chromosomal loci by the one-step gene disruption method (Rothstein 1983).

*Construction of dhh1Δ::URA3*: A 2.5-kbp DNA fragment (*Pma*CI-*Apa*LI) bearing the *DHH1* gene (ORF: 1518 bp) was filled in with Klenow enzyme and subcloned into the *Sma*I site of pBluescript II. The resulting plasmid DNA was digested with *Bst*EII and *Bgl*II. This step deleted 115 bp within the coding region (+438 to + 552). After the ends were filled in with Klenow enzyme, a 1.2-kbp *URA3* fragment, isolated by digestion of YEp24 plasmid DNA with *Hin*dIII and filled in, was inserted. The resulting plasmid was digested with *Pvu*I and *Kpn*I and used for transformation. After heterozygous disruption was confirmed by Southern blotting, the transformants were subjected to sporulation followed by micromanipulation to generate haploid cells carrying the *dhh1* deletion mutation.

*Construction of ccr4Δ::HIS3*: The *ccr4Δ::HIS3* allele was introduced in our background and L40 strain using the TM1 plasmid as described (Malvar *et al.* 1992).

*Construction of pkc1Δ::HIS3*: A 4.3-kbp *Sph*I-*Sph*I fragment bearing the *PKC1* gene was recovered from the original *MPT3* plasmid. Disruption of the *PKC1* gene was performed by the replacement of a 0.6-kbp *Bam*HI fragment of the *PKC1* gene with the *HIS3* gene from pJJ216 as described (Yoshida *et al.* 1992).

*Construction of stm1Δ::ADE8*: A 1600-bp DNA fragment (*Pma*CI-*Cla*I) bearing the *MPT4* gene (ORF: 819 bp) was filled in with Klenow enzyme and subcloned into the *Sma*I site of the pUC18 plasmid whose *Pst*I site was disrupted. The resulting plasmid was digested with *Xba*I and *Hpa*I to remove 33 bp within the *MPT4* gene (+283 to +316) and a 1152 bp *ADE8* DNA whose ends were *Xba*I and *Sma*I. The resulting plasmid was digested with *Eco*RV and *Afl*II, and was used for transformation.

*Construction of mpt5Δ::HIS3*: A 5-kbp *Aat*I-*Aat*I DNA fragment bearing the *MPT5* gene was isolated from the original *MPT5* plasmid DNA, whose ends were changed to *Sal*I by linker ligation, and subcloned into the *Sal*I sited of pUC18. The plasmid DNA was digested with *Nsp*V to remove −420 to +3700 of the *MPT5* DNA. After the ends were filled in with Klenow enzyme, a 1.8-kbp *HIS3* DNA whose ends were filled in with Klenow enzyme was inserted. The resulting plasmid was digested with *Eco*RI and *Eco*RV, and was used for transformation.

All disruptions were introduced into D40 cells, and the disruptions were confirmed by Southern blot analysis. Haploid cells carrying the disruption were recovered by standard microdissection.

**Two-hybrid analysis:** Plasmid pBTM116 (kindly provided by Katsunori Tanaka, Shimane University) was used to construct LexA-Pop2p. *Bam*HI sites were introduced into the DNA encoding the *POP2* protein by PCR, and the resulting DNA fragment was subcloned into the *Bam*HI site of pBMT116 in-frame to LexA. Plasmid pGADGH was purchased from Clontech (Palo Alto, CA) and was used to construct Gal4 activation domain fusion plasmids (Gal4AD-Ccr4 and Gal4AD-Dhh1). Yeast strain L40 was used as the two-hybrid host (Table 1). The activity of β-galacto-sidase was determined as described (Miller 1972). The LexA-Vpu plasmid contains the hydrophilic segment of Vpu (residues 33–81) fused to full-length LexA (Chiang *et al.* 1996). B42-Pop2p contains residues 149–441 of Pop2p fused in frame to the *E. coli*–derived B42 transcriptional activator (Zervos *et al.* 1993). This segment of Pop2p interacts with Ccr4p in the two-hybrid assay and coimmunoprecipitates with Ccr4p (Draper *et al.* 1995; data not shown). B42-Sip1p contains residues 243–500 of Sip1p (Yang *et al.* 1992) fused to B42. LexA-Dhh1 was constructed by removing the complete *DHH1* sequence from the Gal4AD-Dhh1AD fusion using *Bam*HI and *Sal*I and cloning into the *Bam*HI and *Sal*I sites of LexA-202-1 (Cook *et al.* 1994).

**Immunoprecipitation:** Preparation of protein extracts and immunoprecipitation were performed as described (Draper *et al.* 1995). The polyclonal antibody against the Gal4 activa-

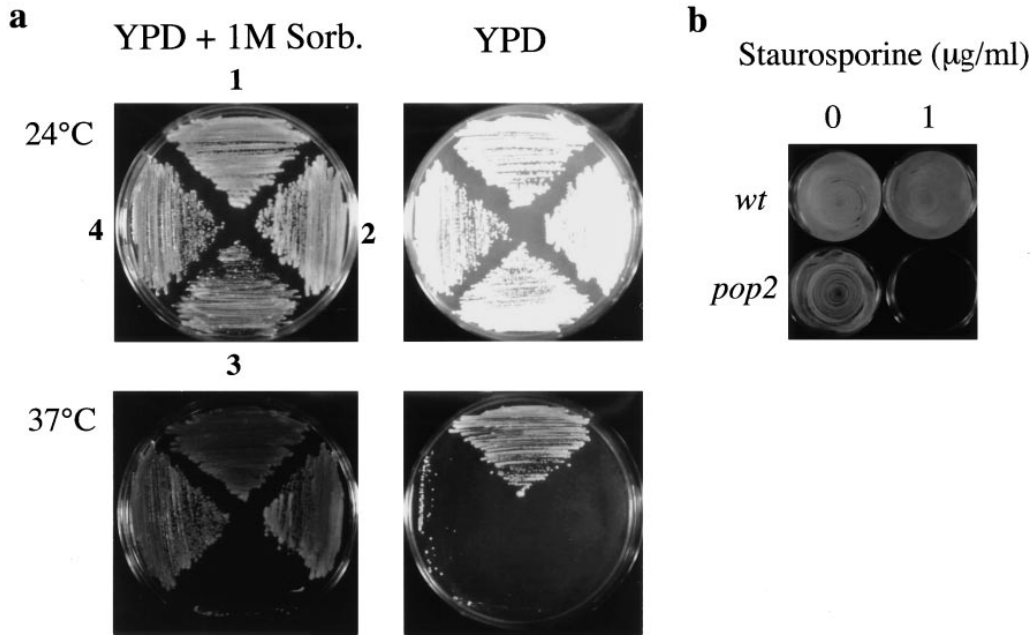


Figure 1.—Newly found phenotypes of *pop2* cells. (a) Sorbitol-suppressible, temperature-sensitive growth defect of *pop2* deletion cells. (1) A1123 (wild-type), (2) A1152 (*stt1-1*/*pkc1*$^{ts}$), (3) A475 (*rgr1-Δ2::URA3*), (4) A880 (*pop2-Δ4::URA3*). Cells were streaked on YPD or YPD+1 M sorbitol plates and were incubated at 24° and 37° for 4 days. (b) Staurosporine sensitivity. A1123 (wild-type) and A880 (*pop2-Δ4::URA3*) cells were resuspended with YPD media, spread on the YPD plate containing staurosporine, and incubated at 24° for 4 days.

**TABLE 2**

**Phenotypes of the deletion mutants**

| | YPD | | | YPD + sorbitol | | YPG | | YPG + sorbitol | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 15° | 24° | 37° | 24° | 37° | 24° | 37° | 24° | 37° | Sta. | Caf. |
| *pop2* | − | + | − | + | + | ±[a] | − | − | − | − | − |
| *ccr4* | − | + | − | + | + | ±[a] | − | − | − | − | − |
| *dhh1* | − | + | − | + | + | ±[a] | − | − | − | − | − |
| *pkc1* | − | − | − | + | − | − | − | − | − | − | − |
| *stm1* | + | + | + | + | + | + | − | + | − | + | + |
| *mpt5* | − | + | − | + | + | + | − | + | − | + | − |

Exponentially growing cells were resuspended in water, and aliquots were spotted on the plate indicated. Plates were incubated at each temperature for 4 days. Plus signs indicate growing (formed colony); minus signs indicate not growing (not formed colony). Sta., 2 μg/ml staurosporine in YPD plate; Caf., 8 mm caffeine in a YPD plate.

[a] These mutants grew slowly on a YPGlycerol, but they did not grow on a CSMGlycerol plate. This phenotype varied depending on genetic background.

tion domain was a kind gift from Kouichi Ishiguro, Mitsubishi Kasei Institute of Life Sciences. The Ccr4p antibody was described previously (Draper *et al.* 1995). Antibodies against the HA1 epitope were commercially obtained. The sequence data presented in this paper have been submitted to the GenBank Data Libraries under the following accession numbers: *MPT4*, D26183; *MPT5*, D26184.

## RESULTS

**Isolation of multicopy suppressors of *pop2*:** The temperature-sensitive phenotype of *pop2* mutants suppressed by the addition of 1 M sorbitol in the medium (Figure 1a). At the restrictive temperature, the *pop2* cells become enlarged and swollen, showing a cell lysis phenotype (data not shown). Furthermore, the *pop2* cells are also sensitive to staurosporine (1 μg/ml; Figure 1b), a potent inhibitor of the yeast protein kinase C homolog (*PKC1*; Tamaoki *et al.* 1986; Levin *et al.* 1990; Yoshida *et al.* 1992). Finally, the growth of *pop2* cells is inhibited by low levels of caffeine (8 mm; Table 2). *pop2* cells also exhibited a weak cold-sensitive growth phenotype that is suppressible by 1 M sorbitol (Liu *et al.* 1997). These phenotypes are similar to those caused by mutations in the *PKC1* pathway, and they suggest that *POP2* plays a role in maintenance of cell wall integrity in addition to its other known phenotypic defects. Since the *pop2* mutant affects such varied processes, it was of interest to us to know the multifunctional roles of Pop2p in cells.

We searched for multicopy suppressors of the temperature- and staurosporine-sensitive growth defects of *pop2* mutation (see materials and methods). We identified five gene products that suppressed the *pop2* phenotypes either completely or partially (Table 3). Nucleotide sequence analysis revealed that these genes are *DHH1*, which encodes a putative RNA helicase (Strahl-Bolsinger and Tanner 1993), *CCR4*, which is required for the full expression of the glucose-repressible *ADH2* gene (Denis and Malvar 1990; Malvar *et al.* 1992), *PKC1*, a yeast homolog of mammalian protein kinase C (Levin *et al.* 1990), *STM1*, a guanine quartet–binding protein (Frantz and Gilbert 1995), and *MPT5* (Kikuchi *et al.* 1994; Kennedy *et al.* 1997). Mpt5p contains eight tandem copies of an ∼38 amino acid–repeat, including a strong consensus sequence of 13 amino acids (LxxDxFGxxFLQK). This repeat is also found in YGL023 (Chen *et al.* 1991) and in the Drosophila pumilio gene (Barker *et al.* 1992; MacDonald 1992). The biological function of this region remains to be identified.

Overexpression of either *CCR4* or *DHH1* suppressed all the *pop2* phenotypes. In contrast, overexpression of *MPT5*, *PKC1*, and *STM* only suppressed some of these

**TABLE 3**

**Summary of multicopy suppression**

| Mutation | | Gene on YEp213 | | | | | |
|---|---|---|---|---|---|---|---|
| | | *POP2* | *CCR4* | *DHH1* | *PKC1* | *STM1* | *MPT5* |
| *pop2* | ts | + | + | + | − | − | + |
| | sta | + | + | + | + | + | − |
| | gly | + | + | + | + | − | + |
| *ccr4* | ts | − | + | + | − | − | + |
| | sta | − | + | + | − | + | + |
| *dhh1* | ts | − | − | + | + | − | − |
| | sta | − | − | + | + | − | − |
| *pkc1* | ts | − | − | − | + | −/+ | − |
| | sta | − | − | − | + | + | − |
| *mpt5* | ts | − | − | − | + | − | + |
| | caf | − | − | − | + | + | + |

Transformants were suspended in water and spotted on YP plates containing either 2% glucose, 2% glucose + 2 μg/ml staurosporine, 2% glucose + 8 mm caffeine, or 2% glycerol. The plates were incubated at 24° or 37° for 4 days. ts, incubated at 37°; sta, 2 μg/ml staurosporine, caf, 8 mm caffeine; gly, 2% glucose.

phenotypes (Table 3). These results suggested a close genetic relationship among *POP2*, *CCR4*, and *DHH1*, while the interaction between *POP2* and *PKC1*, *STM1*, or *MPT5* might be relatively weak.

**Northern analysis:** It is possible that the overexpression of these genes in *pop2* cells suppressed the phenotypic defects if their transcription were being positively regulated by the Pop2. To examine this possibility, we measured the mRNA levels of each suppressor gene in wild-type and *pop2* deletion cells. No significant differences in the mRNA amounts were observed between wild-type and *pop2* deletion cells for any of the suppressor genes (data not shown).

**Genetic interactions among *POP2, CCR4,* and *DHH1*:** Disruptions of *POP2*, *CCR4*, and *DHH1* were constructed (see materials and methods), and the resulting phenotypes were examined. In addition to the phenotypes previously observed for a *CCR4* disruption (Malvar *et al.* 1992; Draper *et al.* 1995; Table 2), we found that in-strain backgrounds other than those of the originally reported, *ccr4*Δ cells are also temperature sensitive for growth, which is suppressed by addition of 1 M sorbitol to the medium. Furthermore, the *ccr4*Δ cells are sensitive to staurosporine and caffeine. While it has been reported that the disruption of *DHH1* does not cause any phenotype (Strahl-Bolsinger and Tanner 1993), we found that *dhh1*Δ cells in our genetic background show a temperature-sensitive cell lysis phenotype that is suppressed by 1 M sorbitol (Table 2). Proteins with RNA helicase activity are often involved in mRNA transport or RNA processing (Margossian and Butow 1996; Dalbadie-McFarland and Abelson 1990). To examine *dhh1* effects on mRNA transport, *dhh1* cells were shifted to 37° for 1 hr, and mRNA accumulation in the nucleus was measured by in situ hybridization with an oligo dT probe. No significant difference was detected between *dhh1* and wild-type cells (data not shown). We also failed to detect pre-mRNA accumulation of the intron-containing *CYH2* gene in *dhh1* cells (T. Tani, personal communication). These observations suggest that the *dhh1* mutation did not affect RNA processing. Cells carrying the *dhh1* mutation also showed cold-sensitive growth at 15°, do not grow on a CSMGlycerol plate, and grow slowly on a YPGlycerol plate. Furthermore, *dhh1*Δ cells are sensitive to staurosporine and caffeine (Table 2, data not shown). Table 4 also shows that *DHH1* is required for the full expression of the *ADH2* gene, a phenotype previously observed with *ccr4* (Denis 1984) or *pop2/caf1*–deleted cells (Draper *et al.* 1995). These phenotypic analyses confirm a close functional link between *POP2*, *CCR4*, and *DHH1*. In contrast, disruption of *PKC1*, *STM1*, and *MPT5* displays only some phenotypic similarities to *pop2*, *ccr4*, and *dhh1* (Table 2) and their mutant phenotypes were consistent with those observed previously (Levin and Bartlett-Heubsh 1992; Frantz and Gilbert 1995; Kikuchi *et al.* 1994; Chen and Kurjan 1997). It is noteworthy that temperature-sensitive growth of *mpt5* cells was suppressed by 1 M sorbitol.

**TABLE 4**

***DHH1* is required for ADH II expression**

| Genotype | ADH II activity (mU/mg) Glucose | Ethanol |
|---|---|---|
| wt | <5 | 2700 |
| *dhh1* | <5 | 550 |

Wild-type (612-1d) or *ddh1* (612-1d-d1) cells were used. Activity of ADH II was determined as described in materials and methods. Standard errors are <15%.

Multicopy plasmids carrying *POP2*, *CCR4*, and *DHH1* were introduced into each mutant in different combinations, and the effects on their growth defects were tested. The *pop2* mutation was suppressed by *CCR4* or *DHH1*, and the *ccr4* mutation was also suppressed by *DHH1* (Table 3); however, the growth defects in *dhh1* cells were not suppressed by either *POP2* or *CCR4*. To further test the epistasis among these genes, cells carrying double mutations were constructed and the phenotypic additivity was tested. We did not find any additive phenotypes in the double-mutant cells, suggesting that *POP2*, *CCR4*, and *DHH1* function in the same pathway, and that *CCR4* and *DHH1* genetically function downstream of *POP2*.

**Dhh1p physically interacts with Pop2p:** The physical association of Dhh1p with Pop2p and Ccr4p was analyzed by two-hybrid analysis and coimmunoprecipitation experiments. The combination of LexA-Pop2 and Gal4AD-Dhh1AD in the two-hybrid system resulted in a significant increase in β-galactosidase activity beyond that obtained with LexA-Pop2p alone (Figure 2, Table 5). LexA-Dhh1p also was observed to interact specifically with a B42-Pop2 fusion, resulting in 18 U/mg of β-galactosidase activity as compared to 2.5 U/mg activity for the interaction of LexA-Dhh1 with B42 alone. Figure 2 shows that residues 147–171 of Pop2p are necessary for the interaction. This region is similar to that which was observed to be necessary for the Pop2p and Ccr4p interaction (Draper *et al.* 1995). Also, disruption of *CCR4* did not abrogate the interaction between LexA-Pop2 and Gal4AD-Dhh1 (Table 5). It should be noted that a *ccr4* disruption reduced the transcriptional activity of LexA-Pop2, which is a general effect that *ccr4* has on LexA activators (Draper *et al.* 1995). We further tested the possibility of an interaction in the two-hybrid system between LexA-Ccr4 and Gal4AD-Dhh1 or B42-Dhh1, and between LexA-Dhh1 and B42-Ccr4, but we were unable to detect any interaction between Ccr4p and Dhh1p, although each of these proteins was expressed in yeast (data not shown). These results suggest that Ccr4p and Dhh1p do not interact directly.

To confirm the physical interaction between Pop2p and Dhh1p, a coimmunoprecipitation experiment was

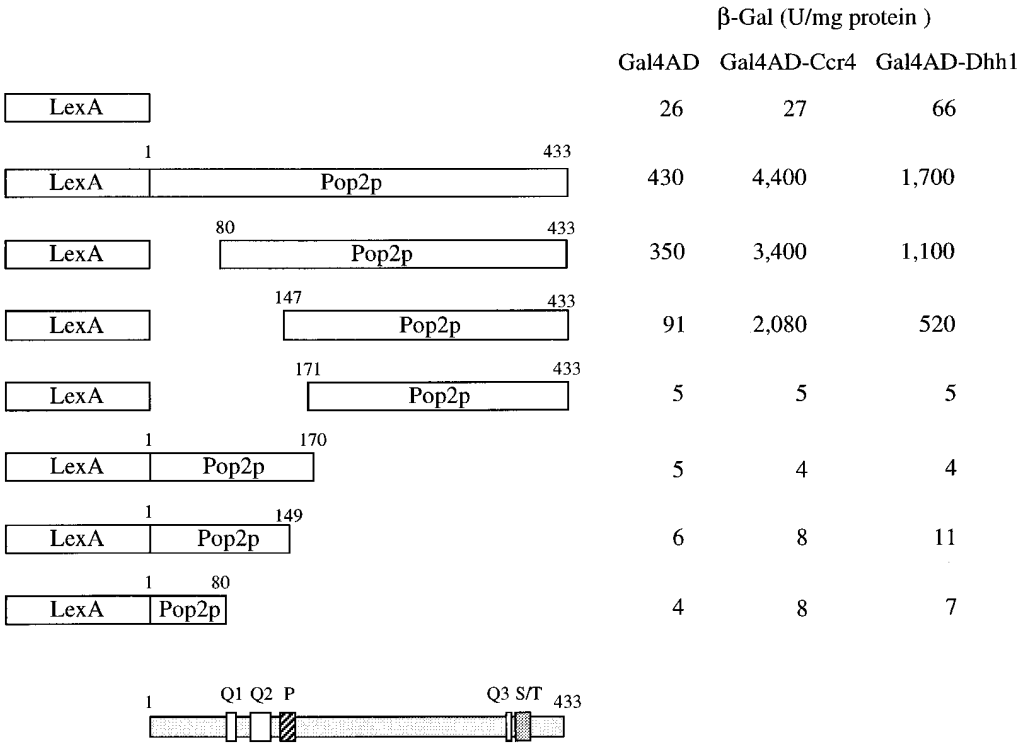


Figure 2.—Region of Pop2p responsible for the interaction with Dhh1p. β-Galactosidase assays and interactions with Gal4p-Dhh1p were conducted as described in materials and methods. Amino acid residues of each segment of Pop2p are indicated. All assays and interactions were done in strain L40. All LexA-Pop2p derivatives were expressed at equivalent levels as assessed by Western analysis (data not shown). Schematic illustration shows location of the characteristic domains. Q1, polyglutamine stretch (residues 81–91); Q2, polyglutamine stretch (residues 112–126); Q3, glutamine-rich region (residues 364–371); P, proline-rich region (residues 139–155); S/T, serine/threonine-rich region (residues 375–391). All the values were the average of at least five independent experiments. Standard errors were <15%.

carried out using transformants carrying LexA-Dhh1 and B42-Pop2 that was tagged with the HA1 epitope (designated HA1-Pop2 in Figure 3). Immunoprecipitating LexA-Dhh1 with anti-LexA antibody resulted in the coimmunoprecipitation of B42-Pop2 (Figure 3, lane 5). The presence of the B42-Pop2 fusion in the immunoprecipitated materials was dependent on LexA-Dhh1 (Figure 3, lane 6). Sip1p, a protein (Yang *et al.* 1992) that is not part of the Ccr4p complex (Liu *et al.* 1997), did not coimmunoprecipitate with the LexA-Dhh1 fusion (Figure 3, lane 4). These results suggest that Dhh1p is physically associated with the CCR4 complex, and that Pop2p can physically interact with both Ccr4p and Dhh1p.

**Genetic interaction between *POP2* and *PKC1-MPK1* pathways:** Because *PKC1* was isolated as a weak multicopy suppressor of the *pop2* mutation, and the phenotypes of *pop2* cells and *mpt* cells are similar to cells carrying mutations involved in the *PKC1-MPK1* pathway, we determined the epistatic relationship among these genes. Overexpression of *POP2*, *CCR4*, or *DHH1* in *pkc1*Δ, *bck1*Δ, *mkk1*Δ *mkk2*Δ, and *mpk1*Δ mutant cells did not suppress any of their phenotypes, including temperature-sensitive growth and caffeine sensitivity. *PKC1* suppressed

**TABLE 5**

**Pop2p interacts with both Ccr4p and Dhh1p in the two-hybrid system**

| Host | DNA-binding hybrid | β-Galactosidase activity (U/mg protein) Activation hybrid | | |
|---|---|---|---|---|
| | | Gal4AD | Gal4AD-Ccr4 | Gal4AD-Dhhl |
| L40 | LexA | 25 | 27 | 61 |
| | LexA-Pop2 | 430 (1.0) | 4,400 (10.2) | 1,700 (4.0) |
| L40/*ccr4* | LexA | 0.7 | 2.4 | 1.2 |
| | LexA-Pop2 | 20 (1.0) | 660 (31) | 67 (3.2) |

Transformants were grown in selective CSM medium. Values are averages of assays of three to five different transformants. Standard errors were <15%. Parentheses indicate fold of induction.

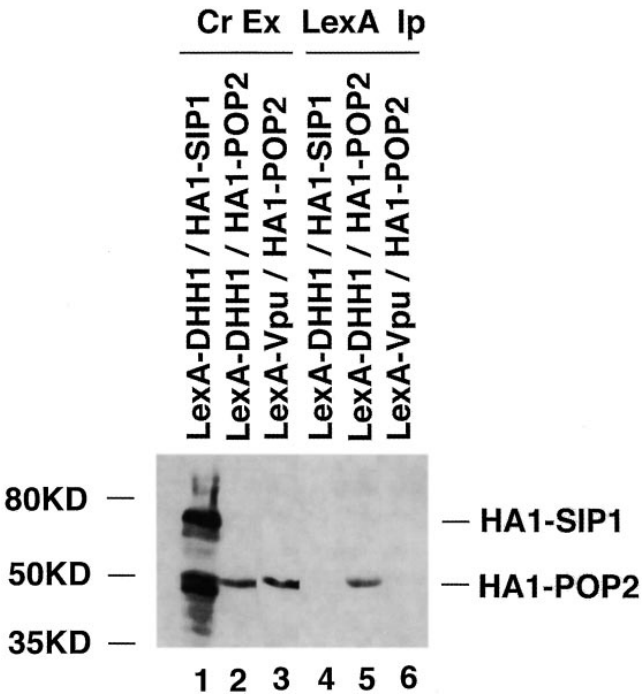


Figure 3.—LexA-Dhh1p coimmuneprecipitates with Pop2p. Crude extracts (lanes 1–3) were prepared from diploid EGY188/EGY191 containing the LexA and B42 (HA1) fusion proteins as indicated. Immunoprecipitations (lanes 4–6) were conducted from these extracts using an antibody directed against LexA. Extracts and immunoprecipitated samples were separated by SDS-PAGE, and proteins were identified by using an antibody directed against the HA1 epitope. LexA-Dhh1p and LexA-Vpu were expressed to comparable extents (data not shown). The protein that comigrates with HA1-Pop2p in lane 1 is a degradation product of HA1-Sip1p and does not coimmunoprecipitate with LexA-Dhh1p (see lane 4).

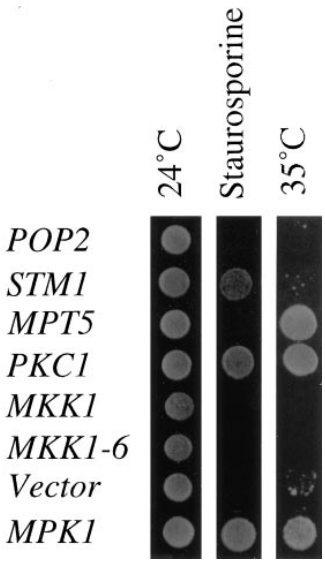


Figure 4.—*PKC1* and *MPT5* suppressed the *mpk1* mutation. Homozygous diploid cells carrying the *mpk1* deletion mutation (DL456) were transformed with YEp213 bearing various genes indicated. The transformants were suspended in water and spotted on YPD plates and a YPD plate containing 2 μg/ml staurosporine. The plates were incubated at 24° or 35° for 4 days. *MKK1-6* encodes a dominant active form of Mkk1p kinase (Lee *et al.* 1993b).

the temperature-sensitive growth of *mpk1* deletion cells at 35° (Figure 4). Overexpression of *STM1* suppressed the staurosporine- and caffeine-sensitive growth, not only of *pop2*Δ mutants, but also of *pkc1*Δ, *bck1*Δ, *mkk1*Δ *mkk2*Δ, or *mpk1*Δ mutants. Thus, *STM1* may function downstream of these genes. *MPT5* overexpression suppressed temperature-sensitive growth defect of *mpk1*Δ deletion cells at 35° (Figure 4), but no other phenotype of this mutant. These results suggest an interaction between the *POP2* and *PKC1-MPK1* pathways, and that Ccr4 and Dhh1 may function downstream of both the *POP2* and *PKC1-MPK1* pathways.

*POP2* and *PKC1* pathways may function independently but have overlapping functions. To examine these possibilities, double-mutants carrying *pop2*Δ *dhh1*Δ, *pop2*Δ *mpt5*Δ, and *dhh1*Δ *mpt5*Δ were constructed. They all showed temperature-sensitive phenotypes. No phenotypic additivity was observed, except that the temperature-sensitive growth of *dhh1*Δ *mpt5*Δ double-mutant cells was not suppressed by 1 M sorbitol (data not shown). On the other hand, haploid cells carrying *pkc1*Δ *pop2*Δ, *pkc1*Δ *dhh1*Δ, or *pkc1*Δ *mpt5*Δ were not recovered. Among the tetrads analyzed (10 for *pop2*Δ and *pkc1*Δ, eight for *dhh1*Δ and *pkc1*Δ, and eight for *mpt5*Δ and *pkc1*Δ), most tetrads contained less than three viable spores, even on the YPD plate containing 1 M sorbitol (data not shown). Judging from the disruption markers, all the dead cells were found to carry either *pop2*Δ *pkc1*Δ, *dhh1*Δ *pkc1*Δ, or *mpt5*Δ *pkc1*Δ. This synthetic lethality displayed phenotypic additivity and suggested that the *POP2* pathway and *PKC1* function independently. Taken together, the phenotypic similarity and genetic suppression among these mutants suggest that *POP2* and *PKC1* pathways function independently but have some overlapping functions.

## DISCUSSION

**Dhh1p, a putative RNA helicase, associates with Pop2p and Ccr4p physically and functionally:** We identified five multicopy suppressor genes of the *pop2* deletion mutation. Two of these, *CCR4* and *DHH1*, appear functionally related to *POP2*. The overexpression of *DHH1* or *CCR4* rescues all the *pop2* defects tested, including high-temperature–sensitive growth, cold-sensitive growth, the inability to use glycerol, and staurosporine and caffeine sensitivity (Tables 2 and 3). Overexpression of *DHH1* also suppresses the growth defects in *ccr4* cells. In addition, *dhh1*Δ cells display a spectrum of phenotypes similar to *pop2*Δ or *ccr4*Δ cells. Importantly, *DHH1* was required for the full expression of the *ADH2* gene (Table 4), another phenotype shared by *pop2* and *ccr4* cells. Furthermore, cells carrying the double mutations *pop2*Δ *ccr4*Δ, *pop2*Δ *dhh1*Δ, or *ccr4*Δ *dhh1*Δ showed no phenotypic additivity (data not shown; Draper *et al.* 1995). These results suggest that there exists a close genetic interaction among the *POP2*, *CCR4*, and *DHH1* genes, and that these three proteins function together to control transcriptional processes. Because Dhh1p is

a putative RNA helicase but no defect in RNA processing was apparent in *dhh1* cells, we think that Dhh1p is involved in a novel aspect of gene expression.

Pop2p and Dhh1p were found to interact physically (Table 5, Figure 3), suggesting that Dhh1p is associated with the Ccr4p regulatory complex. Because we have no evidence to suggest an interaction between Ccr4p and Dhh1p by two-hybrid analysis, it cannot be excluded that Dhh1p interacts with Pop2p in a complex separate from that which associates with Ccr4p. This seems unlikely, however, since Dhh1p has also been found to coimmunoprecipitate with Dbf2p and Caf17p, two other Ccr4p complex components (unpublished observations; Liu *et al.* 1997). More importantly, nearly all the Ccr4p in the cells copurifed with Pop2p (Caf1p; M. Liu, personal communication), suggesting that there is no separate complex. Because there are multiple components of the Ccr4p complex, it is difficult at this point to clearly determine the order and directness of the interactions between these proteins.

Because overexpression of *PKC1*, *STM1*, and *MPT5* suppressed some phenotypes of *pop2*Δ cells, these genes might function further downstream of *POP2* or bypass some Pop2p functions (Table 3). Stm1p has G4 nucleic acid–binding activity (Frantz and Gilbert 1995), and a null allele of the *stm1* mutation does not cause phenotypes, as observed with the other *mpt* mutations. The relationship between the G4 nucleic acid–binding activity and *POP2* function remains to be elucidated. *MPT5* is required for growth at high temperatures and for the recovery from mating factor–induced G1 arrest (Kikuchi *et al.* 1994), and it is involved in yeast cell aging by redistribution of the Sir2p-Sir3p-Sir4p complex from the telomeres to the nucleolus (Kennedy *et al.* 1997). *MPT5* has been shown to interact with Sst2p and Cdc28p, which is involved in the pheromone signaling pathway and the cell cycle control pathway, respectively (Chen and Kurjan 1997). Since the *MPT5* gene is also involved in the stress response, it may be a general suppressor of temperature stress effects.

Since the *pop2*Δ and *mpt5*Δ showed no phenotypic additivity, *POP2* and *MPT5* also appear to function in the same pathway. Based on the facts that overexpression of *DHH1* or *MPT5* can suppress the *ccr4*Δ mutants, whereas overexpression of CCR4 had no effect on *dhh1*Δ or *mpt5*Δ cells (Table 3), we hypothesize that *DHH1* and *MPT5* function genetically downstream of *CCR4*. Overexpression of *DHH1*, however, could not suppress any phenotypes of *mpt5*Δ cells and vice versa. Furthermore, the *dhh1*Δ*mpt5*Δ double-mutant cells showed temperature-sensitive growth, and this defect was not suppressed by 1 M sorbitol (data not shown). These results suggest that *DHH1* and *MPT5* genetically function in two independent pathways that act after *POP2* and *CCR4*.

**Interaction between the *POP2* and *PKC1-MPK1* pathways:** The protein kinase C pathway, including *PKC1*, *BCK1*, *MKK1* or *MKK2*, and *MPK1*, is important for cell wall integrity in yeast (Thevelein 1994). Mutants of *PKC1* require an osmotic stabilizer (*e.g.*, 1 M sorbitol) for survival. Also, cells carrying the *pkc1*Δ or *mpk1*Δ mutation cannot use glycerol as a sole carbon source (Costigan *et al.* 1994, Table 2). This may suggest that the *PKC1-MPK1* pathway activates the transcription of genes that require nonfermentable carbon sources and those that are involved in cell wall integrity. Since *PKC1* was isolated as a multicopy suppressor of *pop2*Δ, and most of the *mpt* mutants including *pop2* itself showed staurosporine-sensitive phenotypes and inability to utilize nonfermentable carbon sources, and the temperature-sensitive cell lysis phenotype of *pop2*Δ and the cold sensitivity phenotypes of *ccr4*, *pop2*(*caf1*), and *dbf2*, were suppressed by 1 M sorbitol (Liu *et al.* 1997), it was of great interest to us to investigate the relationship between the *POP2* pathway and *PKC1* pathway.

Overexpression of *PKC1* suppressed temperature-sensitive growth of *dhh1*Δ or *mpt5*Δ mutants (Table 3). This suggests that *DHH1* and *MPT5* function upstream of *PKC1*. But cells carrying double mutations of *pop2*Δ*pkc1*Δ, *dhh1*Δ *pkc1*Δ, or *mpt5*Δ *pkc1*Δ were synthetically lethal, ruling out a direct and dependent interaction among *PKC1* and *POP2*, *DHH1*, and *MPT5* (data not shown). In addition, the *dhh1*Δ and *mpt5*Δ mutations were suppressed by overproduction of *PKC1* but not by *MPK1* (data not shown), and overproduction of *MPT5* suppressed the *mpk1* mutation (Figure 4). Because epistasis among these genes is unclear, we speculate that the *POP2* pathway and the *PKC1* pathway are two independent pathways that have overlapping functions. To clarify this, we are currently searching for the common target of the two pathways. It should be noted that a well-accepted model of the *PKC1* pathway suggests that *MPK1* is epistatic to *PKC1* (Thevelein 1994). Our finding that overproduction of *PKC1* suppressed the *mpk1* mutation (Figure 4) seems to conflict with this model. It is possible that this might not be a direct effect, or that overproduction of *PKC1* might activate another pathway (Lee *et al.* 1993).

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