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CDC44: a Putative Nucleotide-Binding Protein Required for Cell Cycle Progression That Has Homology to Subunits of Replication Factor C

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To investigate the means by which a cell regulates the progression of the mitotic cell cycle, we characterized *cdc44*, a mutation that causes *Saccharomyces cerevisiae* cells to arrest before mitosis. *CDC44* encodes a 96-kDa basic protein with significant homology to a human protein that binds DNA (PO-GA) and to three subunits of human replication factor C (also called activator 1). The hypothesis that Cdc44p is involved in DNA metabolism is supported by the observations that (i) levels of mitotic recombination suggest elevated rates of DNA damage in *cdc44* mutants and (ii) the cell cycle arrest observed in *cdc44* mutants is alleviated by the DNA damage checkpoint mutations *rad9*, *mec1*, and *mec2*. The predicted amino acid sequence of Cdc44p contains GTPase consensus sites, and mutations in these regions cause a conditional cell cycle arrest. Taken together, these observations suggest that the essential *CDC44* gene may encode the large subunit of yeast replication factor C.

For a dividing cell to faithfully segregate its genetic material to each daughter cell, several complex processes must occur in a precise order. For example, after completion of DNA synthesis, the chromosomes must align along the metaphase plate, attach to the spindle, and move along the spindle to the poles. Each of these individual mechanical events is integrated with the others by regulatory pathways. Thus, to coordinate DNA synthesis and chromosome segregation, the cell must have a system for communicating the information that DNA replication has ended. Imperfections in the DNA lead to cell cycle arrest; in *Saccharomyces cerevisiae*, this arrest is mediated by the product of the *RAD9* gene (61). Similarly, regulatory mechanisms exist to ensure that cytokinesis ordinarily occurs only after chromosome segregation. In *S. cerevisiae*, mutations which disrupt the formation of a mitotic spindle also lead to cell cycle arrest, mediated by the products of the *BUB* and *MAD* genes (24, 29).

Many genes encoding products involved in DNA synthesis and chromosome segregation have been identified by virtue of mutations that cause cells to arrest at a morphologically distinct stage in the cell cycle. Studies of *S. cerevisiae* mutants with heat-sensitive cell division cycle (Cdc) phenotypes have resulted in the identification of several enzymes involved in DNA replication, including DNA ligase (*cdc9* [2]), DNA polymerase α (*cdc17* [31]), and DNA polymerase δ (*cdc2* [51]). Similar screens have also identified some genes required for chromosome segregation, including *CDC31*, which encodes a calcium-binding protein required for spindle pole body duplication (3). Thus, the determination of the nature of the defect in several cell cycle mutants has led to a better understanding of both DNA replication and mitosis.

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To expand upon the knowledge gained by analysis of the heat-sensitive *cdc* mutants, Moir et al. isolated a group of cold-sensitive mutants that exhibit a Cdc arrest at the restrictive temperature of 17°C (34). Initial characterization of the mutants revealed that four (*cdc44*, *cdc45*, *cdc48*, and *cdc51*) arrest as large budded cells with the nucleus in the neck, a terminal morphology that can result from mutations affecting DNA synthesis or chromosome segregation (19). Analysis of bulk DNA synthesis of *cdc44* mutants suggested that they arrest with fully replicated DNA at 17°C (33). However, later analysis of a similar *cdc* mutant (*cdc45*) indicated that this result could have been misleading, since *cdc45* strains also complete DNA replication at 17°C but arrest before the initiation of DNA synthesis at lower temperatures (22).

We present here a detailed analysis of *CDC44*. We find that *CDC44* encodes a protein with homology both to a human DNA-binding protein (PO-GA) and to three subunits of human replication factor C (RF-C). Although *cdc44* mutants arrest with a G_2/M DNA content at the restrictive temperature, levels of mitotic recombination in mutant cells suggest elevated rates of DNA damage. Consistent with this observation, the *cdc44* arrest is alleviated by DNA damage checkpoint mutations such as *rad9*. We suggest that Cdc44p may associate with components of the DNA replication machinery and serve to enhance the efficiency of DNA replication.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are derived from strain S288C, and they are listed in Table 1. Standard genetic techniques were used in constructing the strains (48). The construction of all strains created by integration of plasmid DNA was verified by Southern analysis (52).

YEPD medium is 1% yeast extract, 2% Bacto Peptone, and 2% glucose. Synthetic dextrose (SD) medium contains 6.7 g of Yeast nitrogen base without amino acids (Difco), 20

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype[a]	Source or reference
CH335	*MAT***a** *his4-539 lys2-801 ura3-52*	This laboratory (22a)
CH559	*MAT*α *his4*	D. Botstein (34)
CH608	*MAT***a** *ade2 cdc44-1*	D. Botstein (34)
CH609	*MAT*α *his4 cdc44-1*	D. Botstein (34)
CH1045	*MAT***a** *ade2 ura3-52 cdc44-1*	This study
CH1139	*MAT*α *his3 leu2 ura3*	This laboratory
CH1458	*MAT***a** *ade2 leu2-3,112 ura3-52 cdc44-1* *MAT*α + + *ura3-52* +	This study
CH1461	*MAT***a** *ade2 leu2-3,112 ura3-52* *MAT*α + + *ura3-52*	This study
CH1496	*MAT*α *ade2 leu2-3,112 ura3-52*	This study
CH1578	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 cdc44::LEU2*[b] [pCH1160(CDC44 URA3)]	This study
CH1584	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52*	F. Winston (63a)
CH1587	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 cdc44::LEU2*[b] [pCH1289(CDC44 TRP1)]	This study
CH1806	*MAT***a** *his4-539 lys2-801 ura3-52 CDC44.URA3*[c]	This study
CH1807	*MAT***a** *his4-539 lys2-801 ura3-52 cdc44-5.URA3*[c]	This study
CH1808	*MAT***a** *his4-539 lys2-801 ura3-52 cdc44-8.URA3*[c]	This study
CH1809	*MAT***a** *his4-539 lys2-801 ura3-52 cdc44-9.URA3*[c]	This study
CH1944	*MAT***a** *ade2 leu2-3,112 ura3-52 cdc44::URA3*[b] *MAT*α + + *ura3-52* +	This study
CH1945	*MAT***a** *ade2 leu2-3,112 ura3-52 cdc44::URA3*[b] *MAT*α + + *ura3-52 cdc44-1*	This study
CH1946	*MAT***a** *ade2 leu2-3,112 trp1Δ63 ura3-52 cdc44::LEU2*[b] *MAT*α + *leu2Δ1* + *ura3-52* +	This study
CH1948	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 cdc44::LEU2*[b] [pCH1451(CDC44)]	This study
CH1954	*MAT***a** *ade2 leu2 his4 lys2 ura3-52 CDC44.URA3*[c] *MAT*α + + + + *ura3-52 CDC44.URA3*[c]	This study
CH1956	*MAT***a** *ade2 his4 lys2 ura3-52 cdc44-8::URA3*[c] *MAT*α + *his4* + *ura3-52 cdc44-8::URA3*[c]	This study
CH1967	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 cdc44::LEU2*[b] [pCH1477(CDC44) pCH1289(CDC44 TRP1)]	This study
CH1988	*MAT*α *ade2 leu2 ura3 cdc44-9.URA3*[c]	This study
CH2028	*MAT*α *ura3 rad9::leu2 leu2 trp1 his7 cdc44-9.URA3*[c]	This study
CH2031	*MAT***a** *ade2 leu2 ura3-52 hom3 can1-51* *CDC44* *MAT*α + + *ura3-52* + + *CDC44.URA3*[c]	This study
CH2032	*MAT***a** *ade2 leu2 ura3-52 hom3 can1-51* *cdc44-1* *MAT*α + + *ura3-52* + + *cdc44-8.URA3*[c]	This study
CH2033	*MAT***a** *ade2 leu2 ura3-52 hom3 can1-51* *cdc44-1* *MAT*α + + *ura3-52* + + *CDC44.URA3*[c]	This study
CH2077	*MAT***a** *his4 ura3-52 cdc44-9.URA3*	This study
CH2078	*MAT*α *ura3 cdc44-9.URA3 mec1-1*	
CH2079	*MAT***a** *trp1 lys2 his7 ura3 cdc44-9.URA*[c] *mec2-1*	
LH12021	*MAT***a** *ade1 ade2 gal1 his7 lys2 tyr1 ura1 cdc31*	20
7859-7-4	*MAT***a** *leu2 his7 trp1 ura3 rad9::LEU2*	62

[a] Plasmids are enclosed in brackets.
[b] The *URA3*$^+$ or *LEU2*$^+$ marker in this strain disrupts *CDC44*.
[c] The *URA3*$^+$ marker in this strain is adjacent to but does not disrupt *CDC44*.

g of glucose, and 20 g of Bacto Agar (Difco) per liter of medium. Synthetic complete (SC) medium is SD medium supplemented with 20 mg each of adenine, histidine, and uracil, 60 mg of leucine, 30 mg of lysine, and 20 mg of tryptophan per liter of medium. Sporulation medium is 0.3% potassium acetate in distilled water. 5-Fluoro-orotic acid (5-FOA) plates for the selection of yeast *ura3* mutants (5) were made as described previously (1).

Molecular biology. All standard techniques of molecular biology were performed as described elsewhere (1). To create subclones of several plasmids, exonuclease III digestion (Erase-a-base kit; Promega) was used. The deleted plasmids were religated and transformed into *Escherichia coli*. Plasmids were transformed into *E. coli* DH5α (18), CHb123 [F$^-$ *hadR hadM*$^+$ *pyrF*::Tn*5*Δ(*lac*) *x74 galU galK rpsL trpC9390 leuB600*], or HB101 (7). All transformations of yeast strains were performed by the alkali cation method (26) as modified by Sherman et al. (48). For Northern (RNA) analysis, RNA was prepared from yeast strains by a glass bead-phenol method, and Northern blotting was performed as described by Rose et al. (43); DNA containing the *CDC44* or *TUB2* gene was labeled with ^{32}P and hybridized to a nitrocellulose filter containing total RNA or poly(A)$^+$ RNA from yeast strains. All probes used in Northern and Southern analyses were labeled with ^{32}P (NEN) by random priming (kit by Ambion).

Microscopy. To examine nuclear and spindle morphology and to determine the identity of the mother cell, samples of log-phase cultures were fixed and then stained with 4′,6′-diamidino-2-phenylindole (DAPI) and antitubulin antibodies or with DAPI and Calcofluor (fluorescence brightener; Sigma). In preparation for microscopy, samples were fixed in 3.7% formaldehyde at room temperature for 3 to 24 h and then treated as described previously (41). Samples were visualized by Nomarski imaging or indirect immunofluorescence, using a Zeiss Axioskop microscope. The following antibodies were used for immunofluorescence. For tubulin staining, the primary antibody was 4A1, a mouse monoclo-

nal anti-*Drosophila* tubulin antibody (gift of L. Goldstein), and the secondary antibody was fluorescein-conjugated goat anti-mouse antibody (Accurate Scientific). For β-galactosidase staining, the primary antibody was a mouse monoclonal anti-β-galactosidase antibody (Sigma), and the secondary antibody was the same as for antitubulin staining. The antibodies were diluted immediately before use in phosphate-buffered saline–bovine serum albumin (BSA) (0.1 M KH_2PO_4, 0.4 M K_2HPO_4, 9% NaCl, 0.1% BSA). For staining of bud scars, 1 mg of Calcofluor per ml was used (40).

Strains CH559 (*CDC44*) and CH609 (*cdc44*) were used for determination of spindle and nuclear morphology and for chitin staining. They were incubated at 30°C in YEPD medium until early log phase. The cultures were then split, and half was kept at 30°C and half was shifted to 14°C for 12 h (two generation times). The cells were fixed and stained as described above.

Cloning of *CDC44*. To clone *CDC44*, strain CH1045 (*cdc44-1 ade2 ura3*) was transformed with a yeast genomic library in plasmid YCp50 (39) and screened for transformants able to grow at the restrictive temperature. To verify that the plasmids confer complementation of the cold sensitivity of the strain, the plasmids were isolated from these transformants and retransformed into strain CH1045. Three plasmids (pCH1126, pCH1127, and pCH1128) complement the cold sensitivity of the strain and have overlapping restriction maps.

Mapping. *CDC44* was mapped by hybridizing the ^{32}P-labeled 5.4-kb *Pst*I fragment from a plasmid containing the *CDC44* gene (pCH1127) to a CHROMOblot (Clontech) in which the 16 chromosomes of *S. cerevisiae* were resolved. Autoradiography revealed hybridization with a single band corresponding to chromosome XV. To map the gene within chromosome XV, crosses were performed between strains CH609 (*cdc44-1*) and LH12021 (*cdc31*), and linkage was found between the markers. Additional crosses between strains CH608 (*cdc44-1*) and CH1139 (*his3*) also showed linkage between the markers and thus allowed us to map *CDC44* adjacent to *STE13*. In further confirmation of our mapping data, C. A. Flanagan, D. A. Barnes, and J. Thorner provided plasmid pBRSTE13, which sequence analysis shows consists of plasmid pBR322 with an 8-kb insert of yeast genomic DNA containing both *CDC44* and *STE13*.

Essential gene. Two different disruptions of *CDC44* were used to determine whether *CDC44* is an essential gene. For the first disruption, a 1.5-kb *Nru*I-*Sma*I fragment containing the *URA3* gene was inserted into the blunted *Bgl*II site of *CDC44* to create plasmid pCH1165. The 4.5-kb *Kpn*I-*Bam*HI *cdc44*::*URA3* fragment was then integrated into diploid strain CH1458 (*cdc44-1*/*CDC44*$^+$), creating strain CH1944, and into strain CH1461 (*CDC44*$^+$/*CDC44*$^+$), creating strain CH1945. The diploids were induced to sporulate. Sixteen tetrads were dissected; each produced only two viable spores, and none of the viable spores carried the *URA3* insertion. For the second disruption, a 2-kb *Sph*I-*Bam*HI fragment containing the *LEU2* gene was inserted into a *Bgl*II-*Sph*I gap of *CDC44* to create plasmid pCH1288. The 5-kb *Kpn*I-*Bam*HI *cdc44*::*LEU2* fragment was then integrated into strain CH1584 bearing plasmid pCH1160 (*CDC44*$^+$), to create strain CH1578. Strain CH1578 was then mated to strain CH1496 (*CDC44*$^+$) to produce a diploid, and the diploid was induced to lose plasmid pCH1160 by growth on 5-FOA, creating strain CH1946 (*cdc44*::*LEU2*/*CDC44*$^+$). The diploid was induced to sporulate. Seven tetrads were dissected; each produced only two viable spores, and none of the viable spores carried the *LEU2* disruption. Control diploids not carrying the disruptions produced four viable spores.

Complementation assays. To determine the smallest region of complementing activity in plasmid pCH1127, three sets of subclones were created and tested for the ability to complement the cold sensitivity of strain CH1045 (*cdc44-1*). All subclones were transformed into strain CH1045, and the transformants were examined for the ability to grow on YEPD medium at 30 and 14°C. The first set of subclones (pCH1160, pCH1171, and pCH1159) was created by digesting a yeast centromere plasmid bearing the *URA3* gene (pRS316) (50) with *Kpn*I and *Bam*HI, which cut in the polylinker. A 3-kb *Kpn*I-*Bam*HI fragment from plasmid pCH1127 was ligated into the *Kpn*I-*Bam*HI sites, creating plasmid pCH1160. To create both plasmids pCH1171 and pCH1159, plasmid pCH1160 was first digested with *Hin*dIII and *Sac*I and then incubated with exonuclease for a various times to create a deletion series. Plasmid pCH1171 contains a 1.5-kb 5′ deletion of the *CDC44* open reading frame. Plasmid pCH1159 contains a 1.2-kb 5′ deletion of the *CDC44* open reading frame. To create the second set of subclones (pCH1129 and pCH1282), the 5.4-kb *Pst*I fragment from plasmid pCH1127 that contains the *CDC44* gene was ligated into the *Pst*I site of a yeast centromere plasmid bearing the *TRP1* gene (pRS314) (50), resulting in plasmid pCH1129. A 2.4-kb *Hin*dIII-*Hin*dIII fragment from plasmid pCH1129 was then ligated into the *Hin*dIII site of plasmid pRS316 to create plasmid pCH1282. For the third set of subclones (pCH1283 and pCH1284), two plasmids (p7c and p9c) containing 3′ deletions of *CDC44* created by exonuclease III digestion of pBRSTE13 by Flanagan (15) were used. A 2-kb *Hin*dIII-*Hin*dIII fragment from plasmid p7c was ligated into plasmid pRS316 to create plasmid pCH1283. A 1.8-kb *Hin*dIII-*Hin*dIII fragment from plasmid 9c was ligated into plasmid pRS316 to create plasmid pCH1284.

To compare the abilities of plasmids pCH1160 and a full-length clone of *CDC44* to complement *cdc44-1*, plasmid pCH1496 was created. A 4-kb *Kpn*I-*Xba*I fragment of plasmid pCH1335 (see below, in vitro mutagenesis experiment) was ligated in the presence of a *Kpn*I-*Xba*I-digested pRS316. Instead of integrating into the *Kpn*I-*Xba*I gap of plasmid pRS316, however, restriction analysis indicates that the 4-kb fragment integrated only into the *Kpn*I site, possibly because of incomplete digestion of the vector. Therefore, the entire pRS316 polylinker remains in plasmid pCH1496. Plasmid pCH1160 and plasmid pCH1496 were then transformed into strain CH1045 (*cdc44-1*), and the transformants were found to grow equally well on YEPD medium at 30 and 14°C.

DNA sequencing. The *CDC44* gene was sequenced by the dideoxy sequencing method (47), using a Sequenase reagent kit (U.S. Biochemical Corp.). To prepare the gene for sequencing, a series of 5′ nested deletions of plasmid pCH1160 was created by exonuclease III digestion. In addition, a series of 3′ nested deletions of *CDC44*, created by exonuclease III digestion of pBRSTE13 by Flanagan (15), was used. A series of plasmids containing subclones of *CDC44* were also used for sequencing. The sequences of the primers used are available upon request. The reactions were run on 6% acrylamide gels for 2 h at 55 W. All reported sequence has been determined twice in both directions. Sequence analysis was performed by using the Genetics Computer Group (GCG) sequence analysis software package version 7.1 (12) to search the GenBank data base (4).

To confirm that the 5′ end of the full-length clone, plasmid pCH1308 (see above), contained the same structure as the genomic copy of the gene, Southern analysis of plasmid

pCH1308 and genomic DNA isolated from strain CH559 (*CDC44*$^+$) (1) was performed. The Southern blot was probed with a ^{32}P-labeled 2-kb *Bgl*II fragment isolated from plasmid pCH1308.

Nuclear localization. To obtain inducible *lacZ* fusions of *CDC44* in the cell, P_{GAL}-*lacZ*-*CDC44* fusions were created as described elsewhere (58). A 2.7-kb *Bam*HI *CDC44*$^+$ fragment of plasmid pCH1496 was inserted into the *Bam*HI site of plasmid pUR290 (*lacZ*) (45), 3′ to *lacZ*. A 5-kb *Cla*I fragment containing the 3′ 2.2 kb of *lacZ* fused to *CDC44* was inserted into the *Cla*I site of pMR483 (P_{GAL}-*lacZ*) to create plasmid pCH1451. As a control, a *Cla*I fragment of pUR290 containing *lacZ* was inserted into the *Cla*I site of pMR483 to create plasmid pCH1477, a full-length clone of *lacZ* under the *GAL1* promoter. Plasmids pCH1451 and pCH1477 were transformed into strain CH1587 (*cdc44*::*LEU2 trp1Δ1 ura3*, plasmid pCH1160 [*CDC44*$^+$ *TRP1*$^+$]), resulting in the creation of strain CH1967. The transformants containing plasmid pCH1451 were allowed to lose plasmid pCH1160, resulting in the creation of strain CH1948. Surprisingly, plasmid pCH1451 complements a disruption of *CDC44*, even when the strain is grown under noninducing conditions (data not shown).

To determine the location of the *CDC44* fusion protein, strains CH1967 and CH1948 were grown in YEPRaf until early log phase at 30°C and then shifted into YEPRaf plus 2.5% galactose at 30°C to induce expression of the *GAL1* promoter. Samples were taken 5 h after the shift to galactose medium. The samples were fixed and stained with an anti-β-galactosidase antibody and then visualized by indirect immunofluorescence.

In vitro mutagenesis. To generate plasmids for in vitro mutagenesis (8), plasmid pRS314 was first digested with *Kpn*I and *Bam*HI. A 3-kb *Kpn*I-*Bam*HI fragment from plasmid pCH1127 was isolated and ligated into the *Kpn*I-*Bam*HI sites of plasmid pRS314 to create plasmid pCH1289, a partial-length clone of *CDC44* in a vector bearing *TRP1*. To create a full-length clone for the second plasmid shuffle experiment, plasmid pCH1289 was digested with *Bam*HI. Plasmid pBRSTE13 (gift of C. A. Flanagan, D. A. Barnes, and J. Thorner) was then digested with *Bam*HI, and a 1.1-kb *Bam*HI fragment containing the 5′ end of *CDC44* was isolated and ligated into plasmid pCH1127 to create plasmid pCH1335. To compare the abilities of the partial clone and the full-length clone of *CDC44* to complement a disruption of *CDC44*, strain CH1578 (*cdc44*::*LEU2 trp1Δ1 ura3*, plasmid pCH1160 [*CDC44*$^+$ *URA3*$^+$]) was transformed with plasmid pCH1289 or plasmid pCH1335, and the transformants were plated onto SD medium. The resulting transformants were tested for the ability to remain viable after losing plasmid pCH1160 [*CDC44*$^+$ *URA3*$^+$] by assaying their ability to grow on SC medium supplemented with 5-FOA at 30 and 14°C.

The plasmid shuffle experiments were performed as described previously (8), using plasmids pCH1289 and pCH1335, which were randomly mutagenized in vitro by a 42-h incubation in hydroxylamine (Sigma) (44). The mutagenized plasmids were then transformed into strain CH1578. After eviction of the plasmid carrying wild-type *CDC44* (pCH1160), the transformants were examined for the ability to grow at the restrictive temperature. The nature of the mutation in each allele was determined by DNA sequencing and confirmed by subcloning the mutant segment of the gene into a wild-type *CDC44* plasmid. The sequence of *cdc44-1* was determined by copying the mutation by PCR from the chromosome of a strain (CH1045) carrying the mutation.

To integrate plasmids bearing the mutant or wild-type alleles into the chromosome of *CDC44*$^+$ strains, a 1.5-kb *Nru*I-*Sma*I *URA3*$^+$ fragment was inserted in the *Sna*BI site adjacent to the new *cdc44* alleles. The plasmids were digested with *Kpn*I and *Bam*HI and transformed into strain CH335 to create strains CH1806 (*CDC44*$^+$.URA3$^+$), CH1807 (*cdc44-5.URA3*$^+$), CH1808 (*cdc44-8.URA3*$^+$), and CH1809 (*cdc44-9.URA3*$^+$).

Analysis of the 5′ end of mutant alleles. To create 5′ deletion/*cdc44* double mutants, the full-length mutant plasmids pCH1336 (*cdc44-5*), pCH1350 (*cdc44-8*), and pCH1351 (*cdc44-9*) were digested with *Bam*HI to remove the 1.1-kb *Bam*HI piece at the 5′ end of *CDC44*. The plasmids were religated and transformed into *E. coli*. To create full-length *CDC44* genes containing *cdc44* mutations, the partial-length mutant plasmids pCH1297 (*cdc44-2*), pCH1312 (*cdc44-3*), and pCH1296 (*cdc44-4*) were digested with *Bam*HI. The 1.1-kb *Bam*HI fragment from pBRSTE13 containing the 5′ end of *CDC44* was then inserted, the plasmids were ligated and transformed into *E. coli* as described above, and the orientation of the inserts was verified by digestion with *Bgl*II.

Fluorescence-activated cell sorting (FACS) analysis. Diploid strains CH1956 (*cdc44/cdc44*) and CH1954 (*CDC44*$^+$/*CDC44*$^+$) were incubated at 30°C for 3 h, 14°C for 10 h, or 10°C for 21 h. The samples were fixed, sonicated, and stained with propidium iodide as previously described (25). Stained samples were counted by a Becton Dickinson FACScan fluorescence-activated cell sorter.

Mitotic recombination assay. Mitotic recombination assays were performed essentially as described previously (21). Briefly, the appropriate homozygous and heterozygous *CDC44* diploid strains (CH2031, CH2032, and CH2033) were constructed. Three separate colonies from each strain were inoculated into 5 ml of YPD medium and incubated at 30°C for 18 h. The cultures were split and incubated for a further 2 h at 30°C, 5 h at 21°C, or 12 h at 14°C. Cultures were harvested, sonicated, and plated on SC medium or SC medium containing canavanine (60 μg/ml). After incubation at 30°C for 3 days, the colonies were counted. To test whether canavanine resistance was due to chromosome loss or mitotic recombination, canavanine-resistant colonies were replica plated to test for auxotrophy at the *hom3* locus. In each case, approximately 90% of the canavanine-resistant colonies derived from mitotic recombination and 10% derived from chromosome loss.

First-cycle arrest assays. The method of Weinert and Hartwell (59) was used to determine whether *cdc44*, *cdc44 rad9*, *cdc44 mec1*, and *cdc44 mec2* double mutants arrest in the first cell cycle following a shift to the restrictive temperature. Cells were grown overnight in YEPD medium at 30°C to exponential phase. Cells were then sonicated, diluted, plated on prechilled YEPD plates, and incubated at 14°C for 10 to 24 h. Following incubation, the number of cell bodies (one, two, three, four, or more than four) in 300 microcolonies was determined for each time point. In this assay, cells which undergo a cell cycle arrest in the first division will give rise to microcolonies containing either two or four cell bodies (depending on whether the cell has passed the cell division cycle execution point at the time of the shift to the restrictive temperature). Cells which fail to arrest will give rise to either three or more than four cells. Thus, the number of microcolonies containing either two or four cell bodies divided by the total number of microcolonies scored yields the number of cells which have undergone a first-cycle

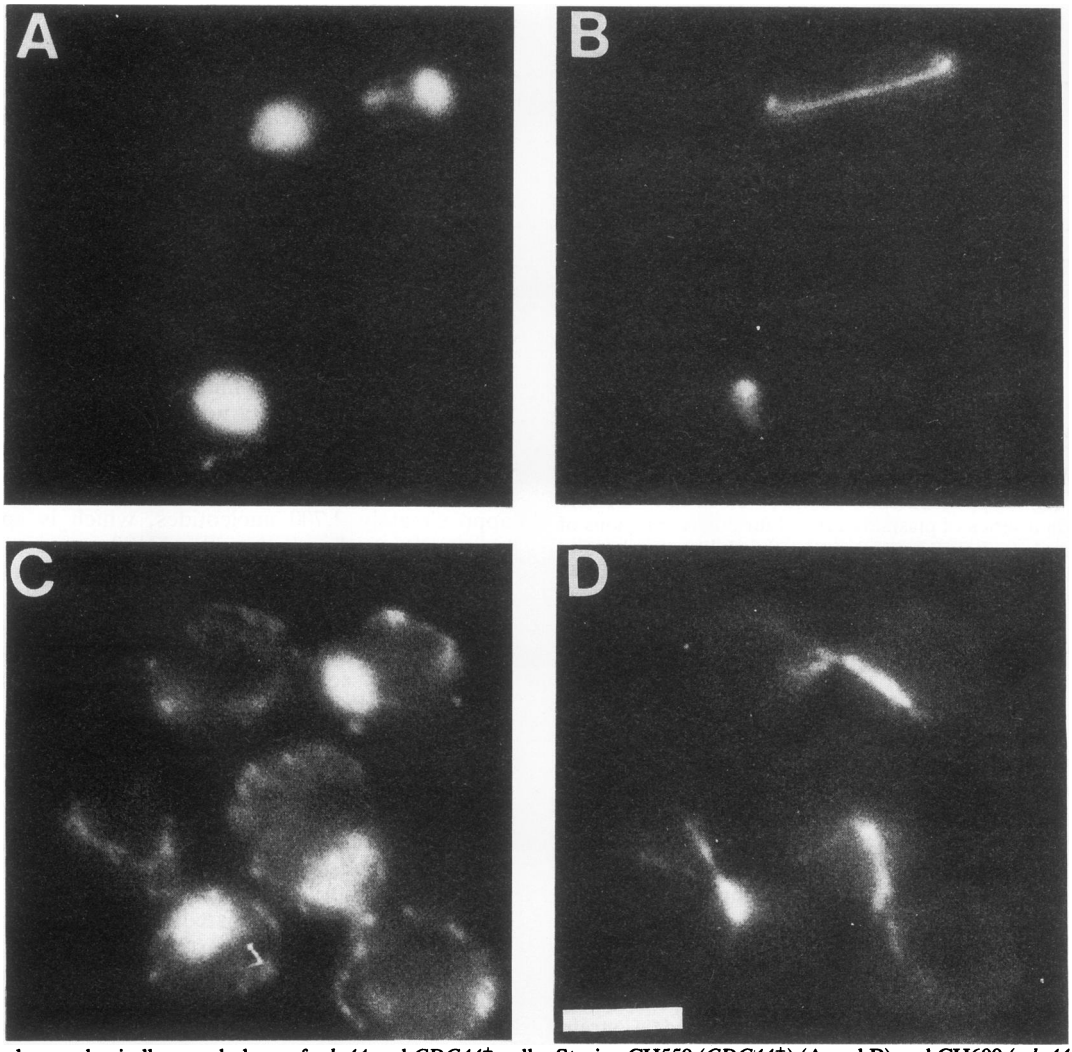

FIG. 1. Nuclear and spindle morphology of *cdc44* and *CDC44*$^+$ cells. Strains CH559 (*CDC44*$^+$) (A and B) and CH609 (*cdc44-1*) (C and D) were incubated at 30°C until early log phase and then shifted to 14°C for 12 h (two generation times). Samples were fixed, stained with DAPI (A and C) or an antitubulin antibody (B and D) and visualized by indirect immunofluorescence. Scale bar = 5 μm.

arrest. The data reported are averages of three independent experiments.

Viability assays. The method of Weinert and Hartwell (59) was used to determine whether *cdc44 rad9*, *cdc44 mec1*, and *cdc44 mec2* double mutants lose viability when incubated at the restrictive temperature. Cells were grown overnight in YEPD medium at 30°C to exponential phase, and they were then shifted to the restrictive temperature (14°C). Aliquots of each culture were removed at intervals, sonicated, diluted, and plated on YEPD plates. After incubation at 30°C for 1 day, cell viability was determined by microscopy; individual microcolonies on each plate were scored as either inviable (fewer than 10 cell bodies) or viable (more than 10 cell bodies).

Nucleotide sequence accession number. The sequence of the *CDC44* gene has been submitted to GenBank and assigned the accession number U03102.

RESULTS

***cdc44-1* mutants arrest as large budded cells prior to nuclear division.** Since the terminal phenotype of a cell division cycle mutant often provides information about the stage in the cell cycle at which the affected gene product is ordinarily required (19), we examined the terminal morphology of strains carrying the *cdc44-1* mutation. *cdc44-1* was originally identified as a mutation that causes cells to arrest at the restrictive temperature with large buds and a single nucleus in the neck between the mother and the bud (34). To extend this observation, we incubated several haploid *CDC44*$^+$ and *cdc44-1* strains at permissive (30°C) and restrictive (14°C) temperatures for two to three generation times and examined the morphology of the nuclei and spindles by fluorescence microscopy. We found that the large budded cells produced at 14°C contain short spindles with numerous cytoplasmic microtubules (Fig. 1). Chitin staining of bud scars to identify the mother cell (40) reveals that the nuclei are found in buds as well as in mother cells (data not shown). This result indicates that *cdc44-1* cells undergo nuclear transits during arrest (38). The terminal morphology produced by *cdc44-1* mutants is similar to the phenotype of mutants that are perturbed in either DNA replication or chromosome segregation (19).

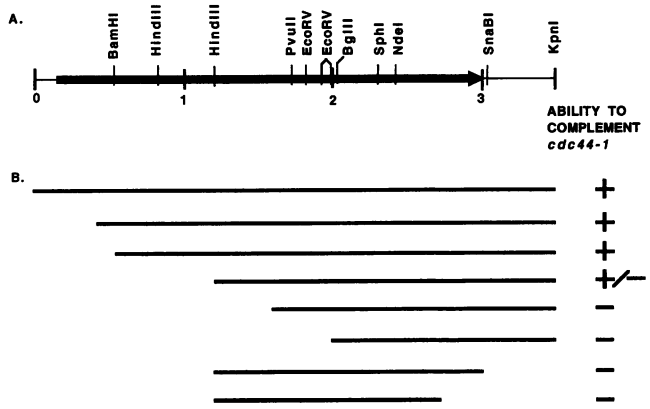

FIG. 2. The 3.5-kb clone containing *cdc44*-complementing activity. (A) the *CDC44* open reading frame (thick line) and flanking genomic DNA (thin line). Arrow indicates predicted direction of transcription. (B) Complementation analysis. Thick lines indicate DNA present in a series of plasmids deleted for different regions of the clone. Complementation was scored as the ability of a deletion plasmid to complement the cold-sensitive phenotype of CH1045 (*cdc44-1*). From top to bottom, the plasmids represented are pCH1496, pCH1127, pCH1160, pCH11282, pCH1159, pCH1171, pCH1283, and pCH1284.

***CDC44* encodes a novel, essential, 861-amino-acid protein with nuclear localization sites.** To examine the nature of the gene product encoded by *CDC44*, we cloned and mapped the gene and determined that the gene product is essential. To clone the gene, we transformed a *cdc44-1* strain (CH1045) with a yeast genomic library (39) and screened for transformants able to grow at the restrictive temperature. Three plasmids (pCH1126, pCH1127, and pCH1128) that complemented the cold sensitivity of the strain were isolated and found to have overlapping restriction maps. To verify that we had cloned *CDC44*, we integrated a plasmid containing the clone (pCH1124) by homology into a *cdc44-1* strain (CH1045) and showed that the integrated plasmid and the mutation are linked (16 of 16 parental ditype tetrads). We mapped the gene to chromosome XV by hybridizing a Southern blot of yeast chromosomes with a probe containing the *CDC44* sequence (data not shown). Crosses between strains carrying *his3*, *cdc44-1*, and *cdc31* show that *CDC44* maps to the right arm of chromosome XV near *STE13* (18 centimorgans between *CDC44* and *CDC31* [16 parental ditype:0 nonparental ditype:9 tetratype] and 13 centimorgans between *CDC44* and *HIS3* [17 parental ditype:0 nonparental ditype:6 tetratype]). A restriction map of a plasmid bearing the *STE13* gene reveals that *CDC44* and *STE13* are adjacent genes (15a). Finally, to examine the effect of a deletion of *CDC44*, we created and sporulated hemizygous diploid strains bearing a either a deletion or a disruption allele of *CDC44* (see Materials and Methods). When the hemizygous diploids are sporulated and dissected, no spores carrying a disruption or deletion of *CDC44* survive (23 tetrads); therefore, *CDC44* is an essential gene.

We delimited the *CDC44* gene by deletion analysis, sequencing, and Northern analysis. First, we created a series of subclones that were deleted for some region of the original 3-kb complementing fragment. When these plasmids were transformed into a *cdc44-1* strain, the cold sensitivity of the strain was complemented by a 2.5-kb *Bam*HI-*Kpn*I segment (Fig. 2). Using these subclones and a larger clone containing all of *CDC44* and *STE13* (plasmid pCH1308), we sequenced the gene to determine the size of the *CDC44* gene product. The DNA sequence reveals a 2,583-bp open reading frame that is predicted to encode an 861-amino-acid protein (Fig. 3). This predicted size is consistent with the size of a fusion protein detected by Western blot (immunoblot) analysis (23). Surprisingly, none of the three clones originally isolated by complementation contains the full-length open reading frame determined by sequencing plasmid pCH1308. However, these partial-length clones complement both *cdc44-1* and a disruption of *CDC44* just as well as a full-length clone created from plasmid pCH1308 does (plasmid pCH1335; see Materials and Methods). Since only one of the cloned segments contains the entire 5′ portion of the open reading frame (plasmid pCH1308), we confirmed by Southern analysis that it contains the same structure of the 5′ region as the genomic copy does (data not shown). To confirm the predicted size of the open reading frame, we probed a Northern blot of poly(A)$^+$ mRNA with the *Hin*dIII-to-*Kpn*I fragment of *CDC44*. We found that *CDC44* encodes a single mRNA of approximately 2,700 nucleotides, which is approximately one-fourth as abundant as the *TUB2* message in exponentially growing cells (data not shown). This message size is consistent with the predicted size of the protein. Finally, an examination of the promoter reveals the sequence TATA 40 bp upstream of the first ATG, which is consistent with the location and sequence of a yeast TATA consensus (53). While many genes whose gene products are involved in DNA replication have an *Mlu*I site consensus sequence upstream of their promoters (17), neither DNA sequencing nor restriction analysis reveals any *Mlu*I sites in the *CDC44* promoter.

Sequence analysis of the predicted *CDC44* gene product reveals a basic (pK_a = 10.0) 96-kDa protein with regions of consensus to nuclear localization sites. Two putative nuclear localization signals are located at the carboxy-terminal end of the protein. A similar bipartite nuclear targeting sequence consisting of two lysine-rich domains separated by a 10-amino-acid spacer region is necessary and sufficient to target nucleoplasmin and N1 to the nucleus in *Xenopus* cells (42). Similar lysine-rich regions also target proteins into the nucleus in yeast cells (16). To determine the cellular location of the *CDC44* gene product, a galactose-inducible *lacZ-CDC44* fusion was introduced into a *cdc44* deletion strain. The fusion protein is localized in the nucleus of cells carrying P_{GAL}-*lacZ-CDC44* grown in the presence of galactose (data not shown). In contrast, cells containing a P_{GAL}-*lacZ* fusion show diffuse overall staining when grown in galactose. (No staining was seen when either strain was grown in the presence of glucose [data not shown].) Although these results could be an artifact of the high expression of the fusion protein, the fact that specific localization occurs even under overexpression conditions suggests that the *CDC44* gene product contains a bona fide nuclear targeting signal.

Sequence analysis and mutagenesis suggest that *CDC44* encodes a nucleotide-binding protein. Further sequence analysis revealed that the predicted *CDC44* gene product contains regions of consensus to nucleotide-binding proteins. The homology to nucleotide-binding proteins occurs in the middle third of the protein. It is difficult to predict solely on the basis of sequence whether a putative nucleotide-binding protein is more likely to be an ATPase or a GTPase. Nonetheless, the sequence of the *CDC44* gene product exhibits homology to all four regions of the GTPase consensus sequence (6), although there is one conservative change in the fourth region (Fig. 4). The conserved sequences also exhibit the expected spacing between the regions, which is

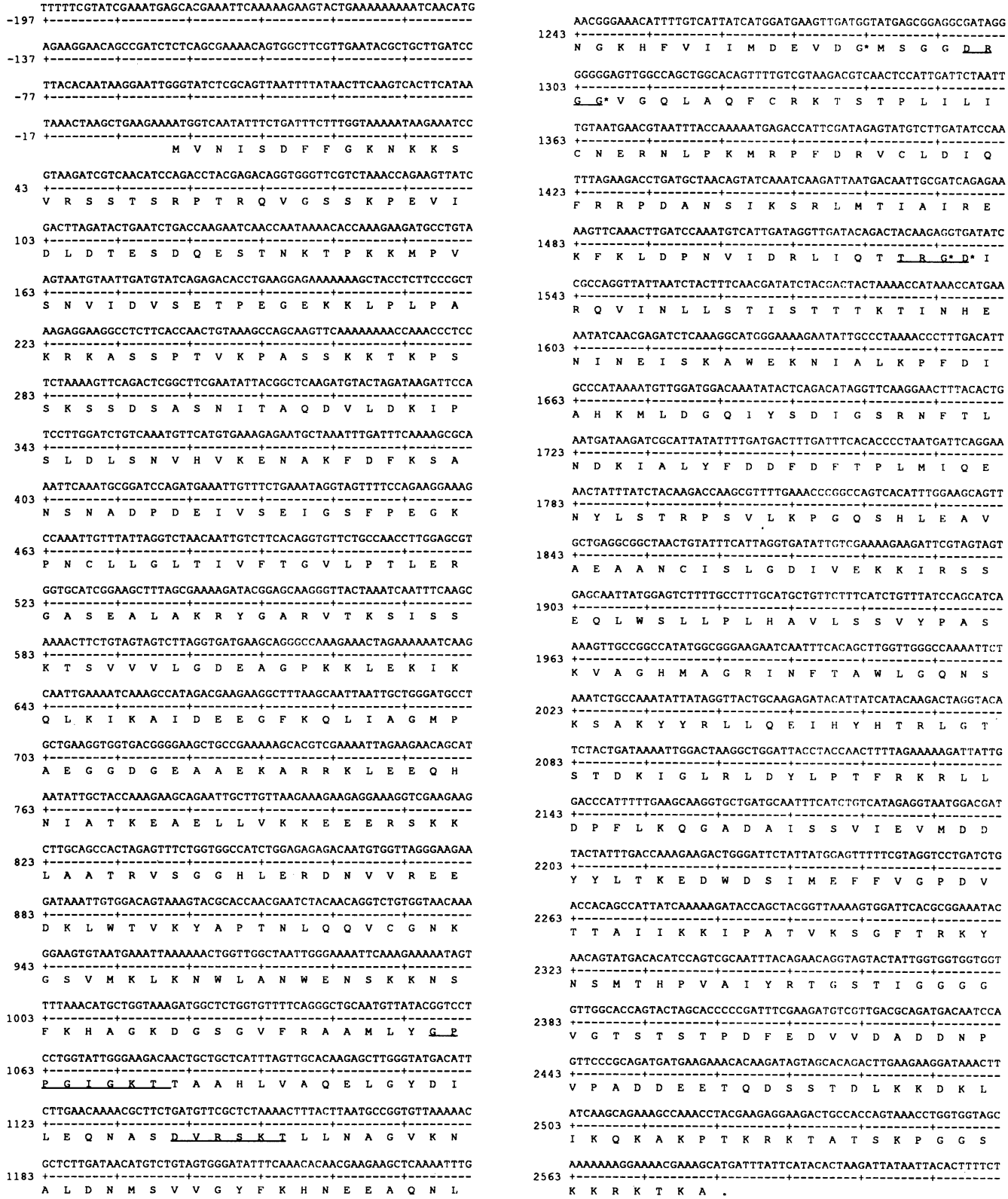

FIG. 3. Nucleotide sequence and predicted amino acid sequence of the *CDC44* gene. The nucleotide sequence of *CDC44* was determined by the dideoxy sequencing method (47). The nucleotide sequence is numbered beginning at the A of the initiator ATG. The predicted amino acid sequence starts with the first methionine in the open reading frame. The period indicates the termination codon. Underlined amino acids indicate the region of homology to the GTPase consensus sequence (6). Asterisks indicate mutation sites of *cdc44-1* (D513N), *cdc44-2* (G428H), *cdc44-4* (G436R), and *cdc44-9* (G512A, D513N).

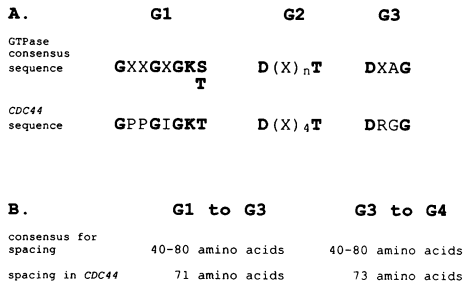

FIG. 4. Comparison of the *CDC44* gene product to a consensus sequence for GTPases. (A) Comparison of the predicted amino acid sequence of the *CDC44* gene product with the GTPase consensus sequence (6). Amino acids which are identical between the two sequences are represented in boldface type. (B) Comparison of the spacing in the *CDC44* gene product with the consensus for spacing in GTPases (11).

estimated to happen by chance very rarely ($P < 1/5,000$ for a 1,000-amino-acid protein [11]). For these reasons, plus the results of mutational analysis (see below), we believe that *CDC44* may encode a GTPase.

To determine the regions of *CDC44* that are most important for cell cycle progression, we performed random in vitro mutagenesis (44) to generate new conditional alleles by the plasmid shuffle method (8). Two separate experiments were performed, using different portions of the gene. In the first experiment, we used a clone (pCH1289) deleted for the first 415 bp of the gene; when present on a plasmid, this clone complements a disruption of *CDC44* as well as does a full-length clone (data not shown). No mutations conferring heat sensitivity were isolated. However, three independent clones containing mutations that confer cold sensitivity (plasmids pCH1297 [*cdc44-2*], pCH1312 [*cdc44-3*], and pCH1296 [*cdc44-4*]) were isolated. DNA sequencing of these new alleles revealed that *cdc44-2* contains a change from guanine to cytosine at bp 1279, resulting in a change from aspartic acid to histidine at amino acid 427. *cdc44-3* and *cdc44-4* are identical, and each has a change from guanine to adenine at bp 1306, resulting in a change from glycine to arginine at amino acid 446. These mutations are in and near the third region of GTPase homology, a position which could plausibly affect GTP binding or hydrolysis; in $p21^{ras}$, the third region of GTPase homology is likely to bind the γ-phosphate of GTP (37). The cellular and nuclear morphologies of strains bearing these three alleles revealed that all arrest with a phenotype similar to that of *cdc44-1* strains at restrictive temperature (data not shown).

In the second mutagenesis experiment, we used a plasmid carrying a full-length clone of *CDC44* (pCH1335). Once again, no mutations conferring heat sensitivity were isolated; these results suggest that *CDC44* may not be able to mutate to heat-sensitive alleles via hydroxylamine mutagenesis. Three independent clones containing mutations that confer strong cold sensitivity (plasmids pCH1336 [*cdc44-5*], pCH1350 [*cdc44-8*], and pCH1351 [*cdc44-9*]) were isolated. The alterations in *cdc44-5* and *cdc44-8* are identical: a change from guanine to adenine at bp 1537, resulting in a change from aspartic acid to asparagine at amino acid 513. This mutation is in the fourth region of GTPase homology; in $p21^{ras}$, the fourth region of GTPase homology is likely to bind the guanine ring of GTP (37). Strikingly, DNA sequencing reveals the same mutation in *cdc44-1*, which was amplified for sequencing from the chromosome of a strain (CH1045) carrying the mutation. *cdc44-9* contains the same mutation as well as an adjacent alteration from guanine to cytosine at bp 1535; this latter mutation results in a change from glycine to alanine at amino acid 512. At the restrictive temperature, the new alleles all cause cells to arrest with a phenotype similar to that of *cdc44-1* strains (data not shown). Since the *cdc44-9* mutant looks identical upon arrest to the strain carrying *cdc44-1*, it appears that the Gly-to-Ala change at bp 1536 may have no effect on function.

It is surprising that our two rounds of mutagenesis yielded nonoverlapping types of mutations, though each round produced multiple independent isolates of particular mutations. One explanation is that the length of the *CDC44* gene used for each experiment could have determined which alleles were isolated, even though plasmid pCH1289 and plasmid pCH1335 complement a disruption of *CDC44* equally well. For example, the mutations isolated in the full-length clone could be too severe to be consistent with viability in combination with a 5′ deletion. To test this hypothesis, we constructed plasmids carrying both the 5′ deletion and either *cdc44-5*, *cdc44-8*, or *cdc44-9*. The plasmids were assayed for the ability to complement a disruption of *CDC44* by transforming strain CH1578 (*cdc44::LEU2 trp1Δ1 ura3*, plasmid pCH1160 [*CDC44 URA3*]) and testing the transformants on 5-FOA medium at 30 and 14°C. Consistent with our hypothesis, these new double mutants fail to complement the disruption allele of *CDC44* at any temperature (data not shown). Similarly, one might imagine that the mutations isolated from the partial-length clone confer a phenotype only in combination with a 5′ deletion of the gene. To test this hypothesis, we restored the 5′ end of the partial-length clones. The plasmids were assayed for the ability to complement a disruption of *CDC44* in strain CH1578 (*cdc44::LEU2*). As expected, the mutations complement the *cdc44* disruption mutation at both 30 and 14°C (data not shown). Taken together, these results demonstrate that the 5′ end of the plasmids used for mutagenesis determined which set of alleles were isolated.

In summary, mutagenesis of the full-length *CDC44* gene produced the striking result of four independently isolated mutations that all contain the same change in the fourth GTPase box. The equivalent change in the GTPase consensus region of $p21^{ras}$ decreases its ability to bind guanine nucleotides by 100-fold (13). Mutagenesis of a partial-length clone resulted in three independent mutations near the third GTPase box. These changes all resulted in morphologically similar cell cycle arrests at the restrictive temperature. Although these results do not definitively reveal whether Cdc44p is a GTPase or an ATPase, they do suggest that an important feature of the *CDC44* gene product could be the ability to bind to guanine nucleotides in vivo. Perturbing this ability may prevent cell cycle progression at the restrictive temperature.

The *CDC44* gene product contains regions of homology to a human DNA-binding protein and to three subunits of HeLa RF-C. A search of protein data bases revealed sequence homology between the *CDC44* gene product and three other types of proteins: prokaryotic DNA ligases, subunits of human RF-C, and a human DNA-binding protein. The homology to the ligases is significant (46), but it is limited to a small region. The *CDC44* gene product shows 38% identity and 60% similarity to *E. coli* DNA ligase over 73 amino acids with one gap; the *CDC44* gene product shows similar homology to *Thermus thermophilus* thermostable DNA ligase. In each case, the *CDC44* gene product is homologous to a region of the ligase that is postulated to bind NAD^+ (54). Since the *CDC44* gene product is not significantly homolo-

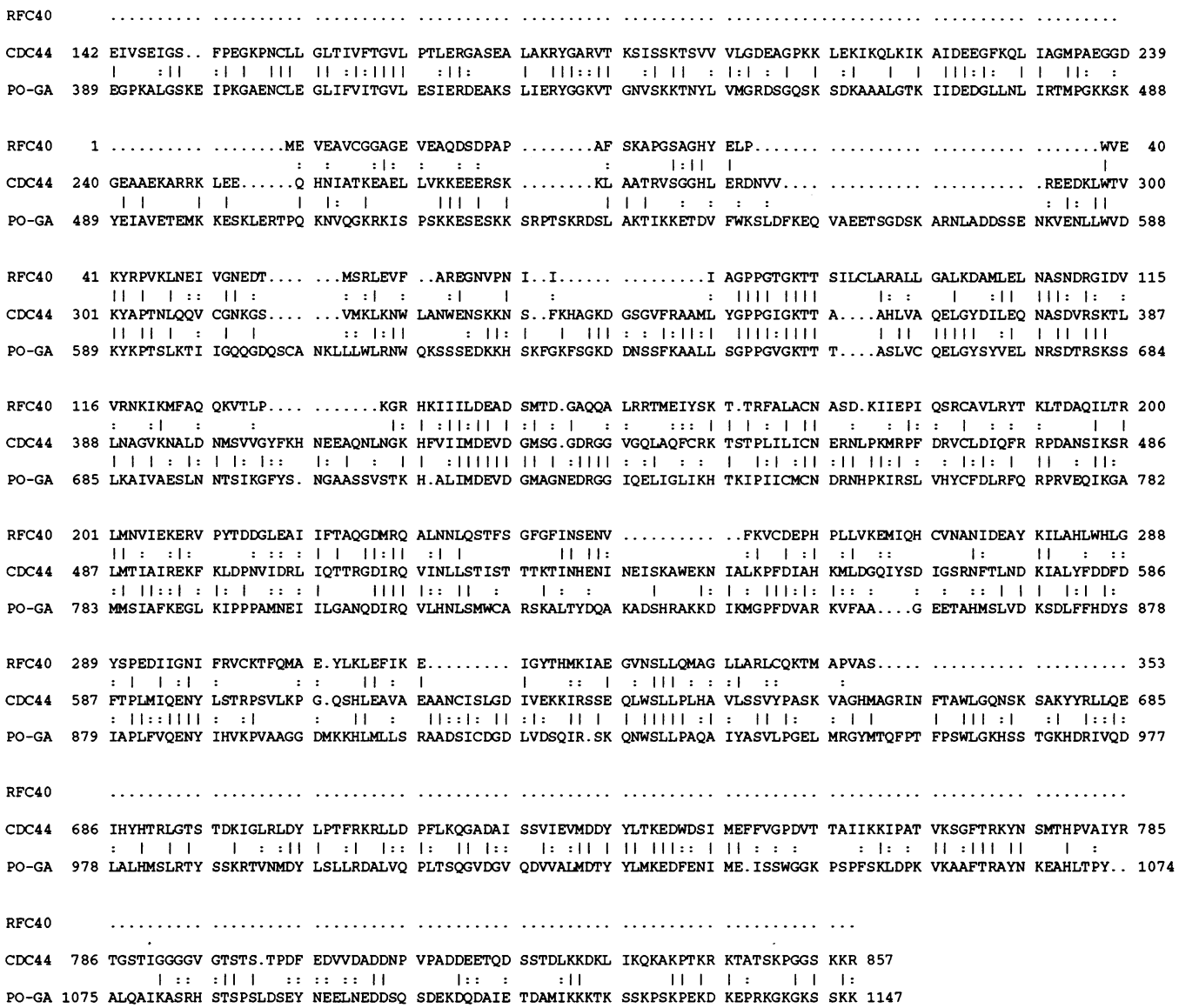

FIG. 5. Sequence comparison of the *CDC44* gene product with the 40-kDa subunit of RF-C (RFC40) and PO-GA. Pairwise alignments of the *CDC44* gene product with RFC40 and with PO-GA were created by using the Genetics Computer Group GAP and BESTFIT programs. Identical amino acids are denoted by vertical lines, and similar amino acids are denoted by colons.

gous to eukaryotic DNA ligases, it is likely that the homology to prokaryotic ligases indicates a putative NAD^+-binding site rather than DNA ligation activity. Further evidence for this hypothesis is provided by examination of the predicted structure of the *CDC44* gene product in the region of homology, which contains a β-sheet, α-helix, β-sheet motif required by the NAD^+ binding site consensus (63).

The *CDC44* gene product exhibits much more extensive homology to the 40-, 37-, and 36-kDa subunits of human RF-C (9, 10, 36). RF-C is a multisubunit complex that interacts with DNA polymerase δ and proliferating cell nuclear antigen (PCNA) (28). Both PCNA and RF-C are required for processive DNA replication by DNA polymerase δ in vitro (55). The homology between the *CDC44* gene product and the RF-C subunits extends over the long central region that includes the nucleotide-binding domains. The *CDC44* gene product and the 40-kDa subunit of RF-C are 27% identical and 48% similar over 186 amino acids with two large gaps and two gaps of one amino acid (Fig. 5). When allowance for a larger number of gaps is included, the homology extends over 410 amino acids. The extent of homology between the *CDC44* gene product and the other small subunits of human RF-C is similar. These homologies are significant, because the homologous regions extend over several hundred amino acids (46).

The most extensive homology occurs between the predicted amino acid sequence of *CDC44* and a human DNA-binding protein called PO-GA (30). PO-GA was originally identified as a protein that binds to the upstream region of the pro-opiomelanocortin gene, and it was subsequently shown to bind to other GA-rich regions of double-stranded and single-stranded DNA (30). Because of sequence homologies and its DNA-binding properties, it has been suggested that PO-GA may play a role in DNA replication or repair

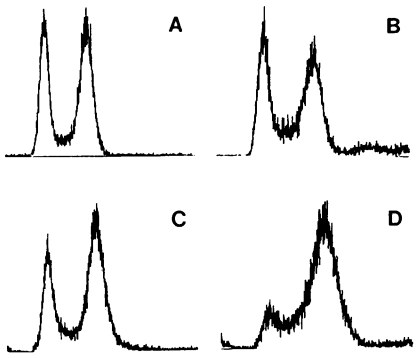

FIG. 6. FACS analysis of *cdc44* and *CDC44*$^+$ strains at the restrictive temperature. Diploid strains CH1956 (*cdc44/cdc44*) and CH1954 (*CDC44*$^+$/*CDC44*$^+$) were incubated at 30°C until early log phase and then shifted to 14°C for 12 h (two generation times) or 30°C for 3 h (two generation times). Samples were fixed, stained with propidium iodide, and counted by a fluorescence-activated cell sorter. (A and B) *CDC44*$^+$/*CDC44*$^+$; (C and D) *cdc44/cdc44*; (A and C) 30°C; (B and D) 14°C.

(30). The homology between the predicted amino acid sequences of the *CDC44* gene product and PO-GA is indeed striking, with 37% identity and 57% similarity over 750 amino acids with 15 gaps (Fig. 5). The *CDC44* gene product and PO-GA may be homologous proteins.

Together with the RF-C homology, the homology with PO-GA raises the possibility that the *CDC44* gene product plays a role in DNA metabolism, perhaps as a component of the replication machinery. It is unlikely that *CDC44* encodes one of the small subunits of yeast RF-C, because yeast RF-C has been isolated, and like human RF-C, it contains at least three subunits of less than 45 kDa (14, 64). There is, however, at least one large subunit of RF-C in both human and yeast cells, and in humans, this subunit has been shown to bind to DNA (57). The predicted molecular size of Cdc44p (96 kDa) is reasonably close to the molecular sizes reported for the large subunit of yeast RF-C (14, 64). Thus, it seems appropriate to speculate that *CDC44* encodes the large subunit of yeast RF-C.

***cdc44* mutants arrest with a G_2/M content of DNA.** DNA sequence analysis suggests that at least one function of Cdc44p may be related to DNA metabolism, and many *cdc* mutants that fail to synthesize DNA at the restrictive temperature arrest with a terminal morphology similar to that observed for *cdc44* (for example, *cdc2* and *cdc7*) (19). To determine the extent of DNA replication in *cdc44* mutants when the cells undergo arrest, we examined the DNA content of *CDC44*$^+$ and *cdc44* strains at the restrictive temperature. We incubated diploid *CDC44*$^+$/*CDC44*$^+$ and *cdc44-1/cdc44-1* strains at the permissive (30°C) or restrictive (14°C) temperature for two generation times and determined the DNA content of the cells by FACS analysis (25). A high percentage of *cdc44/cdc44* cells arrest with a 4N content of DNA at 14°C, indicating that the majority of DNA synthesis can be completed at restrictive temperature (Fig. 6). Similar profiles were observed for these strains when they were incubated at either 21 or 10°C. (Analysis of strains bearing the other mutant alleles [*cdc44-2* or *cdc44-4*] revealed comparable DNA content histograms [data not shown].) This result is not surprising, because many leaky DNA synthesis mutants (e.g., *pol1* [31, 32]) that arrest with a G_2/M content of DNA are known, and our cold-sensitive mutations are not necessarily null mutations. The informative result from the FACS analysis is that these cold-sensitive *cdc44* mutations do not disrupt the bulk of DNA replication at the restrictive temperature.

TABLE 2. Determination of the rates of mitotic recombination for chromosome V

Genotype	Frequency of mitotic recombination events (10^{-4})		
	30°C	21°C	14°C
CDC44/CDC44	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.6
CDC44/cdc44-1	1.2 ± 0.3	1.4 ± 0.4	1.4 ± 0.6
cdc44-1/cdc44-1	19 ± 2.0	23 ± 3.0	26 ± 5.0

[a] Strains CH2031 (*CDC44/CDC44*), CH2032 (*CDC44/cdc44-1*), and CH2033 (*cdc44-1/cdc44-1*) were incubated at 30°C for 18 h, shifted to 30, 21, or 14°C for 1.5 generation times, and assayed for mitotic recombination on chromosome V. Three independent isolates were analyzed for each strain at each temperature.

Although the *cdc44-1* gene product may be sufficient for the bulk of DNA replication at the restrictive temperature, small perturbations in replication or repair in a *cdc44* mutant might be detected by more sensitive methods. We looked for possible perturbations in DNA metabolism by exploiting the observation that various types of DNA damage can lead to an increase in mitotic recombination (21). We assayed *CDC44/CDC44*, *CDC44/cdc44-1*, and *cdc44-1/cdc44-1* diploid strains for levels of mitotic recombination at 30, 21, and 14°C. We found that the level of mitotic recombination was approximately 20-fold higher in *cdc44-1/cdc44-1* cells than in *CDC44/CDC44* or *CDC44/cdc44-1* strains at all temperatures (Table 2). This elevated rate of mitotic recombination suggests that there are increased levels of DNA damage in *cdc44-1/cdc44-1* cells.

The arrest of *cdc44* mutants at the restrictive temperature is relieved by the checkpoint mutations *rad9*, *mec1*, and *mec2*. If *cdc44* mutants undergo cell cycle arrest because of damaged DNA, then the arrest may be mediated by the products of the *RAD9*, *MEC1*, or *MEC2* gene (60, 61). Cell cycle arrest is mediated by the *RAD9* gene product in a variety of cell cycle mutations that produce DNA damage at the restrictive temperature (59). To determine whether the cell cycle arrest in *cdc44* mutants is mediated by the *RAD9* DNA damage checkpoint, we constructed a *cdc44-9 rad9* double mutant strain. We crossed a *cdc44-9 leu2 ura3* strain in which the *cdc44-9* mutation was tagged with an adjacent *URA3* marker (CH1988) with a *rad9 leu2 ura3* strain in which the *rad9* gene was disrupted with a *LEU2* marker (7859-7-4). When the resulting diploid strain was sporulated, analysis of 13 tetrads revealed that all (12 of 12) of the *cdc44-9.URA3 rad9::LEU2* segregants either were inviable or grew poorly on YEPD plates at 30°C. The poor growth was expected, because *cdc44* mutants have elevated levels of DNA damage at the permissive temperature (23).

Strains from these and other crosses were used to examine the dependence of the *cdc44* cell cycle arrest on the *RAD9* gene product. Aliquots of cultures growing exponentially at 30°C were spread on solid medium at 30 or 14°C, and the behavior of the cells over time was observed. Whereas *cdc44-9.URA3 RAD9* strains exhibited 96% first-cycle arrest when incubated at 14°C for 24 h, introduction of the *rad9* mutation in *cdc44-9.URA3 rad9::LEU2* strains reduced the level of arrest to only 48%. Thus, the *rad9* mutation appears to largely alleviate the cell cycle arrest in *cdc44-9* mutants. If this failure to arrest is caused by the removal of a checkpoint, one would expect to observe a concomitant loss of

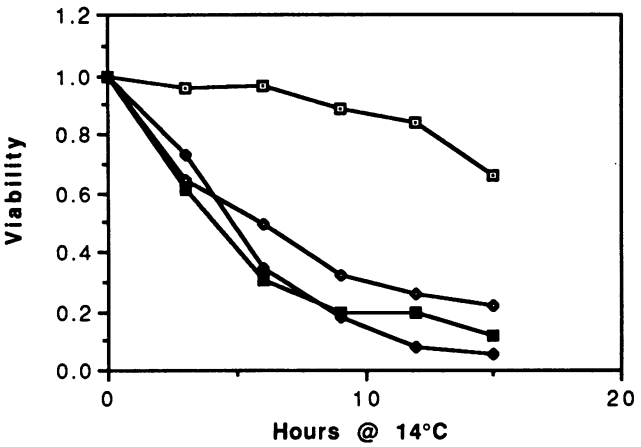

FIG. 7. Viability of double-mutant strains at the restrictive temperature. Strains CH2077 (*cdc44-9*), CH2078 (*cdc44-9 mec1-1*), CH2079 (*cdc44-9 mec2-1*), and CH2028 (*cdc44-9 rad9*) were grown exponentially at the permissive temperature (30°C) and then shifted to the restrictive temperature (14°C). Cell viability was determined at intervals as described in Materials and Methods. The percentage of viable cells in each culture relative to T_0 is presented as a function of the time after the shift to the restrictive temperature. Symbols: ⊡, *cdc44*; ◆, *cdc44 mec1*; ■, *cdc44 mec2*; ◇, *cdc44 rad9*.

viability in the dividing double-mutant strain. Indeed, when viability of the cells is examined in the same experiment, *cdc44-9.URA3 rad9::LEU2* strains exhibit a rapid loss of viability at 14°C (Fig. 7). When similar experiments are performed with *cdc44-9 mec1* and *cdc44-9 mec2* strains, similar results are obtained (33% first-cycle arrest for *cdc44 mec1*, 54% first-cycle arrest for *cdc44 mec2*; Fig. 7). These results are comparable to results observed when a *rad9* mutation is introduced into a *cdc9* (DNA ligase) strain (59). Thus, it appears that the cell cycle arrest in *cdc44* mutant strains is mediated by checkpoint gene products that monitor DNA damage.

DISCUSSION

The data presented here suggest that the *CDC44* gene product plays a role in DNA metabolism. Cold-sensitive alleles of *cdc44* cause cells at the restrictive temperature to arrest with the large doublet morphology that is typical of DNA replication mutants. This arrest is circumvented when the cells are rendered unable to respond to DNA damage by a *rad9* mutation. The cloning and sequencing of *CDC44* reveals a predicted basic protein with significant homology to three subunits of human RF-C, a complex which is required for processive DNA replication in vitro. The *CDC44* gene product also shows excellent colinear homology with the human protein PO-GA, which itself is a DNA-binding protein. Taken together, these results strongly suggest that *CDC44* encodes a gene product that is involved in DNA metabolism.

The presence of the GxxGxGK(S/T) consensus sequence within Cdc44p suggests that the *CDC44* gene product is likely to encode a nucleotide-binding protein. That Cdc44p is a GTPase is suggested by the occurrence of three additional consensus sequences that are associated with GTPases (6, 11). Cdc44p does contain a single mismatch (R instead of K or Q) at the second position within the fourth GTPase consensus box, however. Although this change has not previously been reported within a GTPase, sequence analysis of *cdc44* mutants suggests that these amino acids may not be coincidental matches to the GTPase consensus sequences. The *cdc44-1*, *cdc44-2*, and *cdc44-4* alleles each contain a single mutation that maps within or near the third or fourth GTPase box. In fact, the *cdc44-1* mutation is the same change at the same amino acid position as a mutation in $p21^{ras}$ that lowers GTP binding by 100-fold (13). For these reasons, we believe that Cdc44p is likely to be a GTPase. Demonstration of GTPase or ATPase activity will obviously require biochemical analysis of the purified *CDC44* gene product.

Complementation analysis reveals that the *CDC44* gene product requires neither a complete amino terminus nor regulation by its native promoter to perform its essential function. The original clone of *CDC44* (plasmid pCH1127) is deleted for both the *CDC44* promoter and the first 16 amino acids of the predicted gene product, yet it fully complements a deletion of the gene. A similar result was found when *DBF2* was cloned; the original clone lacks the *DBF2* promoter and the first 75 amino acids of the predicted gene product (27). Sidhu and Bollon found that some bacterial plasmid sequences in pBR322, including a sequence adjacent to the *Bam*HI site, can act as yeast upstream activating sequences (49). Since the YCp50 library contains inserts at the *Bam*HI site, and the *CDC44* open reading frame is in the correct orientation relative to this upstream activating sequence, we believe that *CDC44* transcription is controlled by this promoter in plasmid pCH1127. In addition, a *CDC44-lacZ* fusion under the regulation of the *GAL1* promoter also complements a deletion of *CDC44*, even when the cells are grown on glucose-containing medium. Since the *CDC44* gene product can function properly when its expression is controlled by any of several different promoters, it appears that precise transcriptional regulation is not a critical feature of *CDC44*.

The homology between the *CDC44* gene product and three different subunits of human RF-C suggests that *CDC44* is a member of a multigene family and could be a component of yeast RF-C. RF-C is a protein complex that is required for processive DNA synthesis by DNA polymerase δ in vitro (55). In addition, RF-C exhibits an ATPase activity that is stimulated by another cofactor of DNA polymerase δ, PCNA (56). One model that has gained some credence is that a major function of RF-C is to attach the sliding clamp, PCNA, to the DNA and to DNA polymerase (57). The significance of the homology between Cdc44p and RF-C subunits is underscored by the observation that *cdc44* mutations are suppressed by mutations in yeast PCNA (32a).

Several observations suggest that Cdc44p is likely to be a component of yeast RF-C. First, each of the laboratories that has identified yeast RF-C reports that there is at least one large subunit (14, 64). Although the precise sizes (86, 110, and 130 kDa) do not match the predicted size of Cdc44p (96 kDa), it is not uncommon for migration of a protein on a gel to be somewhat misleading with regard to size (e.g., reference 35). Alternatively, the product of the *CDC44* gene may be modified posttranslationally to give a smaller or larger molecule. Second, it is reasonable to imagine that a mutation affecting RF-C would lead to elevated levels of DNA damage, as *cdc44* mutations do. Finally, like RF-C, the *CDC44* gene product appears to have properties that suggest that it binds directly to DNA. The predicted pK_a of Cdc44p is very basic, and its sequence is quite homologous to a human protein that binds DNA (PO-GA) (30). Taken together, the observed homologies and properties of the predicted *CDC44* gene product suggest that *CDC44* encodes a

protein that plays an essential role in DNA metabolism and is likely to encode the large subunit of yeast RF-C.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York.
2. **Barker, D. G., J. H. M. White, and L. H. Johnston.** 1985. The nucleotide sequence of the DNA ligase gene (*CDC9*) from *Saccharomyces cerevisiae*: a gene which is cell-cycle regulated and induced in response to DNA damage. Nucleic Acids Res. **13:**8323–8337.
3. **Baum, P., C. Furlong, and B. Byers.** 1986. Yeast gene required for spindle pole body duplication: homology of its product with Ca^{2+}-binding proteins. Proc. Natl. Acad. Sci. USA **83:**5512–5516.
4. **Bilofsky, H. S., D. Burks, J. W. Fickett, W. B. Goad, F. I. Lewitter, W. P. Rindone, C. D. Swindell, and C. S. Tung.** 1986. The GenBank genetic sequence database. Gene **14:**1–4.
5. **Boeke, J. D., F. Lacroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylate activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–347.
6. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
8. **Budd, M., and J. L. Campbell.** 1987. Temperature-sensitive mutation in the yeast DNA polymerase I gene. Proc. Natl. Acad. Sci. USA **84:**2838–2842.
9. **Chen, M., Z.-Q. Pan, and J. Hurwitz.** 1992. Sequence and expression in *Escherichia coli* of the 40-kDa subunit of activator 1 (replication factor C) of HeLa cells. Proc. Natl. Acad. Sci. USA **89:**2516–2520.
10. **Chen, M., Z.-Q. Pan, and J. Hurwitz.** 1992. Studies of the cloned 37-kDa subunit of activator 1 (replication factor C) of HeLa cells. Proc. Natl. Acad. Sci. USA **89:**5211–5215.
11. **Dever, T. E., M. J. Glynias, and W. C. Merrick.** 1987. GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. USA **84:**1814–1818.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Feig, L. A., B.-T. Pan, T. M. Roberts, and G. M. Cooper.** 1986. Isolation of *ras* GTP-binding mutants using an *in situ* colony-binding assay. Proc. Natl. Acad. Sci. USA **83:**4607–4611.
14. **Fien, K., and B. Stillman.** 1992. Identification of replication factor C from *Saccharomyces cerevisiae*: a component of the leading-strand DNA replication complex. Mol. Cell. Biol. **12:** 155–163.
15. **Flanagan, C. A.** 1991. Ph.D. thesis. University of California, Berkeley.

15a. **Flanagan, C. A., D. A. Barnes, and J. Thorner.** Personal communication.

16. **Garcia-Bustos, J., J. Heitman, and M. N. Hall.** 1991. Nuclear protein localization. Biochim. Biophys. Acta **1071:**83–101.
17. **Gordon, C. B., and J. L. Campbell.** 1991. A cell-cycle responsive transcriptional element and a negative control element in the gene encoding DNA polymerase α in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **88:**6058–6062.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Hartwell, L. H.** 1976. Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. J. Mol. Biol. **104:**803–817.
20. **Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti.** 1973. Genetic control of the cell division cycle in yeast. V. Genetic analysis of *cdc* mutants. Genetics **74:**267–286.
21. **Hartwell, L. H., and D. Smith.** 1985. Altered fidelity of mitotic chromosome transmission in cell cycle mutants of *S. cerevisiae*. Genetics **110:**381–395.
22. **Hennessy, K. M., A. Lee, E. Chen, and D. Botstein.** 1991. A group of interacting yeast DNA replication genes. Genes Dev. **5:**958–969.

22a. **Holm, C., T. Goto, J. C. Wang, and D. Botstein.** 1985. DNA topoisomerase II is required at the time of mitosis in yeast. Cell **41:**553–563.

23. **Howell, E.** 1993. Ph.D. thesis. Harvard University, Cambridge, Mass.
24. **Hoyt, M. A., L. Totis, and B. T. Roberts.** 1991. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell **66:**507–516.
25. **Hutter, K.-J., and H. E. Eipel.** 1979. Microbial determinations by flow cytometry. J. Gen. Microbiol. **113:**367–375.
26. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
27. **Johnston, L. H., S. L. Eberly, J. W. Chapman, H. Araki, and A. Sugino.** 1990. The product of the *Saccharomyces cerevisiae* cell cycle gene *DBF2* has homology with protein kinases and is periodically expressed in the cell cycle. Mol. Cell. Biol. **10:** 1358–1366.
28. **Lee, S.-H., A. D. Kwong, Z.-Q. Pan, and J. Hurwitz.** 1991. Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase δ. J. Biol. Chem. **266:**594–602.
29. **Li, R., and A. M. Murray.** 1991. Feedback control of mitosis in budding yeast. Cell **66:**519–531.
30. **Lu, Y., A. S. Zeft, and A. T. Riegel.** 1993. Cloning and expression of a novel human DNA binding protein, PO-GA. Biochem. Biophys. Res. Commun. **193:**779–786.
31. **Lucchini, G., M. M. Falconi, A. Pizzagalli, A. Aquilera, H. L. Klein, and P. Plevani.** 1990. Nucleotide sequence and characterization of temperature-sensitive *pol1* mutants of *Saccharomyces cerevisiae*. Gene **90:**99–104.
32. **Lucchini, G., C. Mazza, E. Scacheri, and P. Plevani.** 1988. Genetic mapping of the *Saccharomyces cerevisiae* DNA polymerase I gene and characterization of a *pol1* temperature-sensitive mutant altered in DNA primase-polymerase complex stability. Mol. Gen. Genet. **212:**459.

32a. **McAlear, M. A., E. A. Howell, K. K. Espenshade, and C. Holm.** Submitted for publication.

33. **Moir, D., and D. Botstein.** 1982. Determination of the order of gene function in the yeast nuclear division pathway using *cs* and *ts* mutants. Genetics **100:**565–577.
34. **Moir, D., S. E. Stewart, B. C. Osmond, and D. Botstein.** 1982. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics **100:**547–563.
35. **Morrison, A., H. Araki, A. B. Clark, R. K. Hamatake, and A. Sugino.** 1990. A third essential DNA polymerase in *S. cerevisiae*. Cell **62:**1143–1151.
36. **O'Donnell, M., R. Onrust, F. B. Dean, M. Chen, and J. Hurwitz.** 1993. Homology in accessory proteins of replicative polymerases-*E. coli* to humans. Nucleic Acids Res. **21:**1–3.
37. **Pai, E. F., W. Kabsch, U. Kiengel, K. C. Holmes, J. John, and A. Wittinghofer.** 1989. Structure of the guanine-nucleotide-binding domain of the Ha-*ras* oncogene product p21 in the triphosphate

conformation. Nature (London) **341**:209–214.
38. **Palmer, R. E., M. Koval, and D. Koshland.** 1989. The dynamics of chromosome movement in the budding yeast *Saccharomyces cerevisiae*. J. Cell Biol. **109**:3355–3366.
39. **Penn, M. D., G. Thireos, and H. Greer.** 1984. Temporal analysis of general control of amino acid biosynthesis in *Saccharomyces cerevisiae*: role of positive regulatory genes in initiation and maintenance of mRNA derepression. Mol. Cell. Biol. **4**:520–528.
40. **Pringle, J. R.** 1991. Staining of bud scars and other cell wall chitin with calcofluor. Methods Enzymol. **194**:732–735.
41. **Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer.** 1991. Immunofluorescence methods for yeast. Methods Enzymol. **194**:565–602.
42. **Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64**:615–623.
43. **Rose, M., P. Heiter, and F. Winston.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Rose, M. D., and G. R. Fink.** 1987. *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell **48**:1047–1060.
45. **Rüther, U., and B. Müller-Hill.** 1983. Easy identification of cDNA clones. EMBO J. **2**:1791–1794.
46. **Sander, C., and R. Schneider.** 1991. Database of homology-derived protein structures and the structural meaning of sequence alignment. Proteins **9**:56–68.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
48. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Sidhu, R. S., and A. P. Bollon.** 1990. Bacterial plasmid pBR322 sequences serve as upstream activating sequences in *Saccharomyces cerevisiae*. Yeast **6**:221–229.
50. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**:19–27.
51. **Sitney, K. C., M. E. Budd, and J. L. Campbell.** 1989. DNA polymerase III, a second essential DNA polymerase, is encoded by the *S. cerevisiae CDC2* gene. Cell **56**:599–605.
52. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
53. **Struhl, K.** 1987. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell **49**:295–297.
54. **Tomkinson, A. E., N. F. Totty, M. Ginsburg, and T. Lindhahl.** 1991. Location of the active site for enzyme-adenylate formation in DNA ligases. Proc. Natl. Acad. Sci. USA **88**:400–404.
55. **Tsurimoto, T., and B. Stillman.** 1989. Purification of a cellular replication factor, RF-C, that is required for coordinated synthesis of leading and lagging strands during Simian virus 40 DNA replication in vitro. Mol. Cell. Biol. **9**:609–619.
56. **Tsurimoto, T., and B. Stillman.** 1990. Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. Proc. Natl. Acad. Sci. USA **87**:1023–1027.
57. **Tsurimoto, T., and B. Stillman.** 1991. Replication factors required for SV40 DNA replication in vitro. I. DNA structure specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. J. Biol. Chem. **266**:1950–1960.
58. **Vallen, E. A., T. Y. Scherson, T. Roberts, K. V. Zee, and M. D. Rose.** 1992. Asymmetric mitotic segregation of the yeast spindle pole body. Cell **69**:505–515.
59. **Weinert, T., and L. H. Hartwell.** 1993. Cell cycle arrest of *cdc* mutants and specificity of the *RAD9* checkpoint. Genetics **134**:63–80.
60. **Weinert, T. A.** 1992. Dual cell cycle checkpoints sensitive to chromosome replication and DNA damage in the budding yeast *Saccharomyces cerevisiae*. Radiat. Res. **132**:141–143.
61. **Weinert, T. A., and L. H. Hartwell.** 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science **241**:317–322.
62. **Weinert, T. A., and L. H. Hartwell.** 1990. Characterization of *RAD9* of *Saccharomyces cerevisiae* and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. Genetics **10**:6554–6564.
63. **Wierenga, R. K., P. Terpstra, and W. G. J. Hol.** 1986. Prediction of the occurance of the ADP-binding β-α-β-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187**:101–107.
63a. **Winston, F., and C. Dollard.** Unpublished data.
64. **Yoder, B. L., and P. M. J. Burgers.** 1991. *Saccharomyces cerevisiae* replication factor C I: purification and characterization of its ATPase activity. J. Biol. Chem. **33**:22689–22697.