MOLECULAR AND CELLULAR BIOLOGY, Sept. 1995, p. 4835–4842 Vol. 15, No. 9
0270-7306/95/$04.00+0

Genetic and Biochemical Interactions between an Essential Kinetochore Protein, Cbf2p/Ndc10p, and the *CDC34* Ubiquitin-Conjugating Enzyme

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Received 6 April 1995/Returned for modification 25 May 1995/Accepted 5 June 1995

***CBF2*/*NDC10*/*CTF14* encodes the 110-kDa subunit of CBF3, a key component of the yeast centromere/kinetochore. Overexpression of yeast *CDC34* specifically suppresses the temperature-sensitive growth phenotype of the *ndc10-1* mutation. Mutations in *CDC34*, which specifies a ubiquitin-conjugating enzyme, arrest yeast cells in the G_1 phase of the cell cycle, with no intact spindles formed (M. G. Goebl, J. Yochem, S. Jentsch, J. P. McGrath, A. Varshavsky, and B. Byers, Science 241:1331–1335, 1988). The *cdc34-2* mutation drastically alters the pattern of Cbf2p modification. Results of experiments using antibodies against Cbf2p and ubiquitin indicate that Cbf2p is ubiquitinated in vivo. Purified Cdc34p catalyzes the formation of Cbf2p-monoubiquitin conjugate in vitro. These data suggest that Cbf2p is an endogenous substrate of the *CDC34* ubiquitin-conjugating enzyme and imply that ubiquitination of a kinetochore protein plays a regulatory role in kinetochore function.**

The centromere, a constricted region on the eukaryotic chromosome, contains a complex macromolecular structure (the kinetochore) that interacts with the mitotic spindle, thus bringing about proper chromosome segregation during mitosis and meiosis (5). In the yeast *Saccharomyces cerevisiae*, the minimal DNA sequence required in *cis* for full mitotic and meiotic centromere function is contained within a 125-bp segment (*CEN* DNA) that is organized into three conserved DNA elements, termed CDEI, CDEII, and CDEIII (9, 13). The helix-loop-helix protein CBF1 (also known as CPF1 or CP1) binds to the CDEI region of *CEN* DNA (4, 8, 32). Deletion of CDEI or the single gene specifying CBF1 leads to only a partial loss of centromere function (8). CDEIII, on the other hand, is crucial for proper centromere function, since chromosomes containing single base changes in the central CCG of the palindromic CDEIII sequence are extremely unstable and segregate virtually as acentric chromosomes during mitosis and meiosis (39, 44). Therefore, proteins that bind to this critical CDEIII sequence may play a key role in centromere/kinetochore function. The multisubunit protein complex CBF3 binds specifically to the essential CDEIII region (36). *CEN* DNA affinity-purified CBF3 consists of three major subunits (110, 64, and 58 kDa), while other proteins are usually present in substoichiometric amounts (36).

The genes that code for the three CBF3 core subunits have been cloned and shown to be essential for the viability of yeast cells. *CBF2*/*NDC10*/*CTF14* encodes the 110-kDa subunit of the CBF3 complex (16, 23, 30). The deduced amino acid sequence of Cbf2p contains a motif typical of the consensus GTP-binding domain of various G proteins (30). It is likely that Cbf2p functions on the centromere/kinetochore in vivo, since cells bearing a conditional *ndc10* mutation show a pronounced chromosome segregation defect at nonpermissive temperatures (23). Furthermore, in vivo immunolocalization experiments suggest that Cbf2p is associated with the mitotic spindle and the spindle pole bodies (23, 29).

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The gene (*CBF3*) specifying the 64-kDa subunit of CBF3 has been cloned recently (35). This subunit most likely is directly involved in the CBF3-*CEN* DNA interaction, since anti-Cbf3p antibodies prevent the formation of the CBF3-*CEN* DNA complex when preincubated with CBF3, and in addition, Cbf3p contains a type of zinc finger domain known to be responsible for the DNA binding activities of various transcriptional activators (33, 35). Finally, the 58-kDa subunit of CBF3 is encoded by another essential gene, *CTF13* (16). Temperature-sensitive *ctf13* mutants missegregate chromosomes at permissive temperatures and transiently arrest at nonpermissive temperatures as large-budded cells with a G_2 DNA content and a short spindle, implying that Ctf13p is involved in proper segregation of yeast chromosomes (16). Ctf13p shows no significant amino acid sequence homologies with known proteins.

A clue to the molecular function of CBF3 and associated proteins may be afforded by the finding that affinity-purified CBF3 preparations contain a mechanochemical motor activity. The CBF3 complex can mediate attachment of wild-type (but not mutationally inactivated) *CEN* DNA-coated microbeads to microtubules and, in the presence of ATP, can move the beads at about 4 to 5 μm/min in a minus-end-oriented direction along the microtubules (26). The motor protein has been identified as Kar3p, a yeast kinesin known to be involved in karyogamy and mitosis (40, 42). Both the core CBF3 complex and Kar3p are required for this in vitro microtubule-dependent kinetochore motor activity (42). In addition, crude yeast extracts contain another activity that, in the presence of functional CBF3, mediates binding of *CEN* DNA-coated microbeads to bovine microtubules (52).

To identify other structural and regulatory components of the yeast kinetochore, we have looked for genes that in high dosage can suppress the growth defect in the mutant *cbf2*/*ndc10* gene. One of these dosage suppressors was identified as the yeast gene *MCK1*, which encodes a phosphotyrosyl protein with protein kinase activity (14, 31). *MCK1* has also been identified as a dosage suppressor of partially inactivating point mutations in CDEIII and also of the meiotic block resulting from *RMEI* expression (43, 48). Purified *MCK1* protein kinase

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

Strain	Genotype	Source
HY342	*MAT***a** *his3 ura3 cdc34-2*::*CDC34-URA3*	This work
HY13	*MAT***a** *ade2 leu2-3,112 his3 lys2 trp1 ura3-52 ndc10-1*	Segregant from JK421 × SEY6210
JK421	*MAT***a** *ade1 ade2 leu2-3,112 his lys2 trp1-1 gal1 ndc10-1*	J. Kilmartin
MGG15	*MAT***a** *his3 ura3 cdc34-2*	M. Goebl
E3-16	*MAT***a** *ade1 ade2 ura1 his7 tyr1 gal1 cdc34-1*	L. Hartwell
SEY6210	*MAT*α *leu2-3,112 his3-Δ200 lys2-801 suc2-Δ9 trp1-Δ901 ura3-52*	S. D. Emr
SEY6210.5	*MAT***a**/*MAT*α *ADE2/ade2-101 leu2-3,112/leu2-3,112 his3-Δ200/his3-Δ200 LYS2/lys2-801 SUC2/suc2-Δ9 trp1-Δ901/trp1-Δ901 ura3-52/ura3-52*	S. D. Emr
MW169	*MAT*α *leu2-3,112 lys trp1-Δ1 ura3-52 ndc10-169*	E. A. Siewert and M. Winey
MW209	*MAT***a** *leu2-3,112 his3-Δ206 ura3-52 ndc10-209*	E. A. Siewert and M. Winey

phosphorylates Cbf2p on both serine and threonine residues in vitro (31).

We report here on another *cbf2/ndc10* dosage suppressor, *CDC34*, which specifies a ubiquitin-conjugating enzyme (UBC3) in yeast cells (22). The ubiquitin pathway in eukaryotes is generally involved in selective degradation of damaged or short-lived proteins; a multiubiquitin chain formed on a target protein serves as a degradation signal for the ubiquitin-dependent proteolytic pathway (for reviews, see references 12, 25, and 54). However, a monoubiquitin conjugate formed on a protein is, in many cases, not involved in targeting the substrate for degradation (12). In *S. cerevisiae*, protein ubiquitination is catalyzed by at least 10 different ubiquitin-conjugating enzymes, or E2s. The phenotypes of yeast *ubc* mutants suggest that ubiquitin conjugation of target proteins is required for a variety of cellular functions, including cell cycle progression (reviewed in reference 18). Yeast *CDC34* is essential for cell division; at nonpermissive temperatures, conditional mutants arrest at the G_1/S boundary (7, 22). Here we present evidence indicating that Cbf2p is ubiquitinated in vivo and serves as an in vitro substrate of the *CDC34* ubiquitin-conjugating enzyme. These observations point for the first time to molecular events linking control of kinetochore function and the cell cycle.

MATERIALS AND METHODS

Yeast strains and cell culture. The *S. cerevisiae* strains used in this study are listed in Table 1. Strain MGG15 (*ura3 cdc34-2*) was used to construct the isogenic *CDC34* wild-type strain HY342. Briefly, the 2.6-kb *Bgl*II DNA fragment containing the wild-type *CDC34* sequence was subcloned into the *Bam*HI site of pRS306 (50). The resulting plasmid was linearized at the unique *Bam*HI site present in the middle of the *CDC34* open reading frame to direct plasmid integration to the chromosomal region homologous to the cut sequence. The mutant *cdc34-2* cells were transformed with these linear DNA molecules and incubated at the nonpermissive temperature, 37°C, on plates lacking uracil. The Ts^+ Ura^+ transformants were then checked under the microscope to confirm the wild-type phenotype. *S. cerevisiae* cultures were grown in YEPD, or to maintain selection for plasmids, cells were grown in synthetic minimal medium supplemented with the appropriate amino acids and/or bases. All yeast media used are described in reference 47. Yeast transformations were performed by the lithium acetate method (27), modified as described by Elble (17).

Plasmids. The *CDC34*-containing plasmid was isolated as a dosage suppressor of *ndc10-1* by transforming strain JK421 (*ura3-52 ndc10-1*) with a yeast genomic library constructed in YEp24 (10), selecting for growth at the nonpermissive temperature (35°C). To construct either high-copy-number or low-copy-number plasmids containing *CDC34*, a 2.6-kb *Bgl*II DNA fragment isolated from the original suppressor plasmid was ligated into the *Bam*HI site of YEp24 or pRS316. YEp24 is a 2μm-based vector, and pRS316 is a centromere-based vector (3, 50). Plasmids pWJ13-110B (YEp13 derivative) and pWJ316-110B (pRS316 derivative), described previously (30), were used as high-copy-number and low-copy-number *CBF2* expression plasmids, respectively.

Yeast crude nuclear extracts and immunoprecipitation. Crude nuclear extracts were prepared from various *S. cerevisiae* strains as described by Lechner and Carbon (36). Anti-Cbf2p and antiubiquitin immunoprecipitates were prepared as follows. Ten microliters of affinity-purified anti-Cbf2p antibody (0.5 μg) and 10 μl of protein A-Sepharose (100 mg/ml; Sigma) suspension were incubated for 1 h at 4°C in the presence of 500 μl of buffer A (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [Sigma], 2.5 μg of leupeptin [Sigma] per ml, 2.5 μg of pepstatin [Sigma] per ml). The protein A-beads were then collected by centrifugation (1 min at 8,000 rpm) and resuspended with 500 μl of crude nuclear extract (200 μg of protein) prepared from 30 ml of yeast culture grown to an A_{595} of 1 to 2. After 1 h at 4°C, the pellets containing immune complexes were recovered by centrifugation (1 min at 8,000 rpm) and washed three times in buffer A, twice in buffer B (500 mM LiCl, 100 mM Tris [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 2.5 μg of leupeptin per ml, 2.5 μg of pepstatin per ml), and finally once in buffer A. Antiubiquitin immunoprecipitates were prepared in the same way except that 20 μl of antiubiquitin antibody (about 40 ng) was used. The antiubiquitin antibody was purchased from Sigma (U-5379) and reconstituted according to the manufacturer's instructions. Characterization of affinity-purified anti-Cbf2p antibody has been reported previously (30).

Western blot (immunoblot) analysis. Proteins (crude nuclear extracts, immune complexes, or *CEN* DNA affinity-purified CBF3 preparations) were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. After 1 h of preincubation in buffer C (10 mM Tris [pH 7.4], 50 mM NaCl, 0.1% Tween 20) plus 10% nonfat dry milk, the blots were further incubated with an affinity-purified anti-Cbf2p (final dilution, 1:1,000) or antiubiquitin (final dilution, 1:500) antibody prepared in buffer C plus 5% nonfat dry milk, washed several times with buffer C plus 0.5% Triton X-100, and visualized by enhanced chemiluminescence as instructed by the manufacturer (Amersham).

Ubiquitin in vitro conjugation assay. The assay for Cbf2p-ubiquitin conjugation was carried out in 30 μl of assay buffer which contained 50 mM Tris (pH 7.5), 2 mM ATP, 10 mM $MgCl_2$, 1.2 mM dithiothreitol, 10 mM creatine phosphate, and 14 μg of creatine phosphokinase per ml in the presence of 50 pmol of ^{125}I-labeled ubiquitin (10^5 cpm), 0.1 μg of Cbf2p, 4 pmol of the purified calf thymus ubiquitin-activating enzyme (E1), and 4 pmol of the yeast Cdc34 enzyme. All reagents except Cbf2p were kindly provided by A. Barnerjee and V. Chau (Wayne State University). Cbf2p used in the assay was obtained by immunoprecipitating the protein from Cbf2p-overproducing yeast nuclear extracts as described above. The reaction mixture was incubated at 30°C for 30 min and spun briefly to separate the supernatant from the pellet containing Cbf2p immune complexes. The supernatant was saved for SDS-PAGE, while the pellet was washed three times in buffer A, once in buffer B, and once again in buffer A before analysis by SDS-PAGE. The supernatant and the pellet were then boiled for 5 min in an SDS sample buffer, resolved by SDS-PAGE (7.5% polyacrylamide gel), and subjected to autoradiography.

RESULTS

***CDC34* is a dosage suppressor of the *ndc10-1* mutation.** It is highly likely that yeast kinetochore function depends on structural and regulatory components in addition to CBF1 and the core CBF3 complex. To identify gene products interacting directly or indirectly with the 110-kDa subunit of CBF3, we have looked for yeast genes that when overexpressed can rescue the conditional *ndc10-1* mutation at the nonpermissive temperature. The *ndc10-1* strain was transformed with a yeast genomic library constructed in the 2μm-based high-copy-number plasmid YEp24 (10). The transformants were incubated at 25°C for 2 days and then at 35°C for 5 days on plates lacking uracil. Of 4,000 transformants screened, 5 were capable of growing at 35°C. Demonstration of loss of the rescue phenotype with plasmid segregation and reproducible suppression by isolated plasmids were the criteria used for selecting putative suppressor clones. Only two of the five candidate clones met

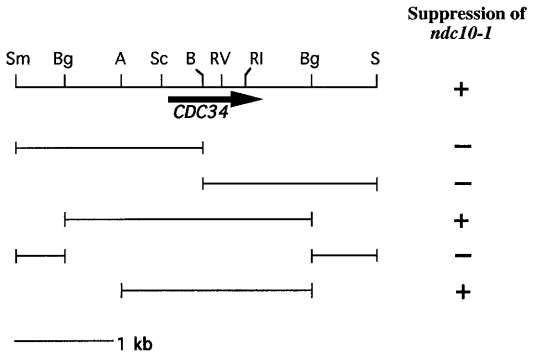

FIG. 1. Restriction map of the *CDC34* region and localization of *CDC34*. The top line represents the primary isolate from the YEp24 genomic DNA library. The arrow indicates the *CDC34* open reading frame. Other line segments indicate DNA fragments derived from the *CDC34* locus. The suppression ability of the DNA fragments was tested at 35°C. Restriction endonuclease sites: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I; Sm, *Sma*I. *Sal*I and *Sma*I sites originated from the YEp24 vector.

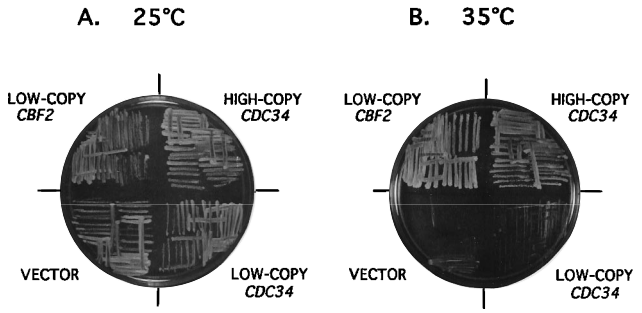

FIG. 2. Dosage suppression of the *ndc10-1* mutation by *CDC34*. Four independent transformants of the *ndc10-1* mutant strain (HY13; JK421 derivative) were streaked on two plates lacking uracil at the same time and incubated for 2 days at 25 or 35°C. The transformants carried the *CBF2* gene on a low-copy-number plasmid (pWJ316-110B), vector alone (pRS316), or the *CDC34* gene on a low-copy-number plasmid (pRS316 derivative) or on a high-copy-number plasmid (YEp24 derivative).

these criteria. One of these suppressors was identified as the yeast *MCK1* gene in work reported elsewhere (31).

The restriction map of the DNA insert in the plasmid isolated from the other suppressed *ndc10-1* strain is shown in Fig. 1. Deletion analysis showed that a plasmid containing the 2.6-kb *Bgl*II fragment suppressed the growth defect of the *ndc10-1* mutant strain at 35°C. The nucleotide sequences determined from both sides of the *Bgl*II and *Bam*HI sites were then used to search the GenBank and EMBL databases for homology, using the FASTA program of the University of Wisconsin Genetics Computer Group, Madison, Wis. One complete open reading frame specifying *CDC34* and the N-terminal portion of *DBF4* could be localized within the 2.6-kb *Bgl*II fragment. Neither the 2.3-kb *Sma*I-*Bam*HI DNA fragment nor the 2.2-kb *Bam*HI-*Sal*I DNA fragment was able to complement the *ndc10-1* mutation, confirming that *CDC34* was the suppressor (Fig. 1). The 2.3-kb *Sma*I-*Bam*HI fragment contains the N-terminal half of *CDC34*, and the 2.2-kb *Bam*HI-*Sal*I fragment carries the C-terminal half of *CDC34*. Subsequent subcloning of a 2.0-kb *Apa*I-*Bgl*II fragment verified *CDC34* as the functional suppressor (Fig. 1).

The suppression was seen only in *ndc10-1* cells bearing a high-copy-number *CDC34* plasmid; the same DNA fragment in a low-copy-number *CEN* plasmid (pRS316) did not suppress the growth defect of *ndc10-1* at 35°C (Fig. 2). High dosage of *CDC34* showed no obvious phenotype in *NDC10* wild-type cells (data not shown). Overexpression of *CBF2* failed to suppress the *cdc34-2* mutation at the nonpermissive temperature, 37°C (data not shown). We tested two other *ndc10* alleles (*ndc10-169* and *ndc10-209*) to see if the suppression was allele specific. While *ndc10-1* and *ndc10-169* cells contain intact spindles after being shifted to the nonpermissive temperature, *ndc10-209* cells show no intact spindles when the mutant cells were incubated for more than 6 h at the nonpermissive temperature (49). As shown in Fig. 3, a high-copy-number plasmid containing *CDC34* suppressed the growth defect of *ndc10-1* cells, but not *ndc10-169* and *ndc10-209* cells, at 35°C (compare sector 2 with sectors 5 and 8). We failed to detect any temperature at which suppression occurred for *ndc10-169* and *ndc10-209* cells. Furthermore, high dosage of *CDC34* was unable to restore the viability of *ndc10-1* cells at temperatures higher than 35°C, whereas a plasmid harboring *CBF2* complemented the mutant phenotype under the same conditions (data not shown). The lethality of a *cbf2* null mutation was not suppressed by overexpression of *CDC34*. Apparently, overexpression of *CDC34* cannot suppress loss of all of the functions supplied by Cbf2p.

The *cdc34-2* mutation affects posttranslational modification of Cbf2p. In immunoblot analyses of yeast crude nuclear extracts, we observed reproducibly that an affinity-purified anti-Cbf2p antibody recognizes higher-molecular-weight protein species in addition to the predicted band at the 110-kDa position (see the protein bands indicated by the dots in Fig. 4). The Cbf2p band appears as a close doublet at about the 110-kDa position, and two other strongly cross-reacting bands with reduced mobilities are apparent (Fig. 4). We believe that these bands represent multiple modified forms of Cbf2p recognized specifically by the anti-Cbf2p antibody, since all of the bands were shifted upward (approximately 4 kDa) on immunoblots of extracts containing a Gal4p activation domain-Cbf2p fusion protein (data not shown). Furthermore, the anti-Cbf2p antibody used in this study was affinity purified over a recombinant Cbf2p-agarose column (30).

Some of the Cbf2p-derived proteins with apparent molecular masses greater than 110 kDa could be phosphorylated forms of the protein. However, it is unlikely that phosphory-

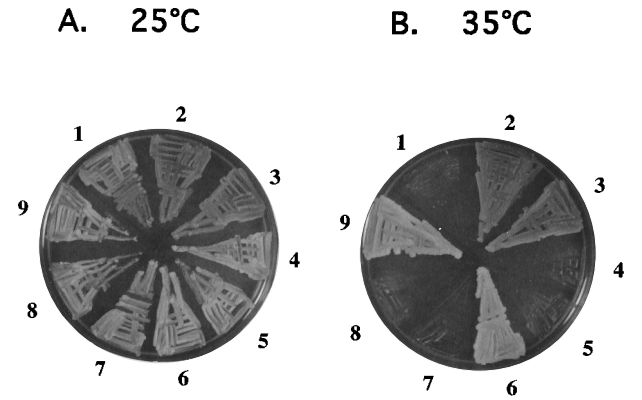

FIG. 3. The *CDC34* suppression is specific to the *ndc10-1* allele. To test the allele specificity of *CDC34* suppression, three different *ndc10* alleles (*ndc10-1*, sectors 1 to 3; *ndc10-169*, sectors 4 to 6; and *ndc10-209*, sectors 7 to 9) were transformed with either vector alone (pRS316; sectors 1, 4, and 7), a plasmid containing *CDC34* (YEp24 derivative; sectors 2, 5, and 8), or plasmid pWJ316-110B (sectors 3, 6, and 9). Single colonies were picked from each transformation and checked for complementation of the temperature-sensitive growth phenotype of the mutant alleles as described in the legend to Fig. 2.

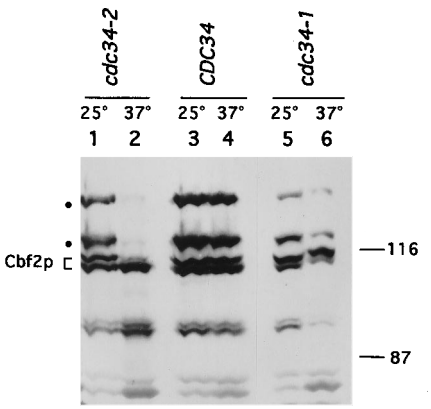

FIG. 4. The *cdc34-2* mutation alters the pattern of Cbf2p modification. Wild-type (strain HY342), *cdc34-2* (strain MGG15), and *cdc34-1* (strain E3-16) cells were grown at 25°C in 100 ml of YEPD medium to an A_{595} of 0.5. The cultures of each strain were divided into two equal aliquots and further incubated at 25 or 37°C for 5 h. Both *cdc34-2* and *cdc34-1* cells showed the typical terminal phenotype when shifted to the nonpermissive temperature. Crude nuclear extracts (40 μg of protein per lane) prepared from these cultures were analyzed by SDS-PAGE (7.5% polyacrylamide gel and subjected to immunoblot analysis. The blot was stained with an affinity-purified anti-Cbf2p antibody (dilution of 1:1,000) and visualized by chemiluminescence as described in Materials and Methods. Dots indicate high-molecular-weight Cbf2p-derived forms. The bracket indicates a close doublet of Cbf2p occurring at about the 110-kDa position. Positions of molecular weight standards (SDS-7B; Sigma) are shown on the right in kilodaltons. The electrophoresis was continued until the 46-kDa colored marker (rainbow marker; Amersham) reached the bottom of the gel.

lation alone could account for the presence of all of the anti-Cbf2p cross-reacting bands with retarded electrophoretic mobilities. Since *CDC34* encodes a ubiquitin-conjugating enzyme, we hypothesized that some or all of the high-molecular-weight species could be ubiquitinated or polyubiquitinated forms of Cbf2p and that Cdc34p, either alone or in conjunction with other ubiquitin-conjugating enzymes, catalyzes the ubiquitination of Cbf2p.

Immunoblot analyses of crude nuclear extracts were first performed to see if overexpression of *CDC34* in the *ndc10-1* strain caused any significant change in levels of the various Cbf2p species, including the more slowly migrating forms. No detectable alteration in levels of the Cbf2p species was observed when the dosage of *CDC34* was increased in either *NDC10* wild-type or *ndc10-1* mutant cells, at both permissive and nonpermissive temperatures (data not shown). Thus, the dosage suppression of the *ndc10-1* mutation by *CDC34* apparently is not reflected in changes in steady-state levels of the Cbf2p species. The effect of inactivating Cdc34p on appearance of the more slowly migrating forms of Cbf2p was then determined by immunoblot analysis of crude nuclear extracts prepared from wild-type, *cdc34-2*, and *cdc34-1* strains. Cells bearing the *cdc34-2* temperature-sensitive mutation are defective in *CDC34*-dependent ubiquitin-conjugating activity at the nonpermissive temperature, 37°C (53). As shown in Fig. 4, the presence of the *cdc34-2* mutation in *CBF2* wild-type cells dramatically altered the pattern of Cbf2p modification when cells grown at the permissive temperature were incubated at 37°C for 5 h. Incubation of *cdc34-2* cells at the nonpermissive temperature drastically reduced the amount of the more slowly migrating Cbf2p-specific bands (Fig. 4, lane 2) in comparison with extracts prepared from the same strain incubated at 25°C (Fig. 4, lane 1). When extracts were prepared from wild-type *CDC34* cells (the wild-type and *cdc34-2* strains were isogenic; see Materials and Methods), no differences in the pattern of Cbf2p-specific bands were induced by incubation of the cells at 37°C (Fig. 4, lanes 3 and 4). Extracts prepared from another mutant allele (*cdc34-1*) showed only partial loss of the more slowly migrating Cbf2p bands at 37°C (Fig. 4, lanes 5 and 6). The *cdc34-1* and *cdc34-2* mutants show the same terminal phenotype under restrictive conditions; both develop multiple elongated buds, and the spindle pole body duplicates but fails to undergo the separation required for spindle formation (7, 22). Thus, the effects on Cbf2p modification seen in the *cdc34-2* strain at 37°C do not arise simply from arrest of cells at G_1/S but are more specific to the *cdc34-2* mutation. Inactivation of *CDC4*, which also results in G_1/S arrest, or a gene encoding another ubiquitin-conjugating enzyme, *UBC2*/*RAD6* (28), had no effect on the apparent ubiquitination of Cbf2p (data not shown).

Cbf2p is ubiquitinated in vivo. To determine if the Cbf2p-specific proteins with molecular masses greater than 110 kDa are modified by ubiquitination, we immunoprecipitated crude nuclear extracts prepared from wild-type yeast cells with either an antiubiquitin or anti-Cbf2p antibody and subjected the immunoprecipitates to Western blot analysis (Fig. 5A). By probing the blots of antiubiquitin and anti-Cbf2p immunoprecipitates with both antibodies, we determined that the antiubiquitin antibody recognizes some of the higher-molecular-weight modified forms of Cbf2p and also the band migrating near the predicted molecular weight of 110 kDa (Fig. 5A, lanes 2 and 4). Immunoprecipitations were performed in the presence of *N*-ethylmaleimide (NEM), since it has been reported that Cdc34p-ubiquitin conjugates are detectable only if yeast extracts were immunoprecipitated in the presence of NEM (21), which inactivates many ubiquitin-dependent proteases and ubiquitin hydrolases (reviewed in reference 19). We do not believe that the antiubiquitin antibody is cross-reacting nonspecifically with the Cbf2p-derived proteins. The addition of NEM during the course of immunoprecipitation significantly changes the apparent pattern of Cbf2p ubiquitination, and the results are essentially the same when the initial immunoprecipitation is carried out with either antibody preparation (Fig. 5A). Also, the antiubiquitin antibody apparently does not cross-react with unmodified Cbf2p, since no signal is seen on a Western blot of Cbf2p isolated from *CEN* DNA affinity-purified CBF3, while the same antiubiquitin antibody cross-reacts with many proteins present in the flowthrough fraction from the *CEN* DNA-affinity column (Fig. 5C). We assume therefore that Cbf2p is not ubiquitinated in the *CEN* DNA-CBF3 complex and that antiubiquitin antibodies specifically interact with ubiquitinated forms of Cbf2p. Since CBF3 must be phosphorylated for *CEN* DNA binding to occur and Cbf2p is modified by phosphorylation (31, 36), phosphorylation could be involved in posttranslational modification of Cbf2p function on the kinetochore.

The intensity of the ubiquitinated Cbf2p bands is much less in the immunoprecipitates than in direct Western blots of crude nuclear extracts (compare bands marked with dots in Fig. 5B with those in Fig. 4, for example). Apparently, the multiubiquitinated forms are unstable and are degraded in immunoprecipitates, even in the presence of NEM (note the predominant degradation product marked Cbf2-D in Fig. 5B). As mentioned above, the antiubiquitin antibody recognizes a Cbf2p band occurring at about the 110-kDa position (Fig. 5A, arrow). When anti-Cbf2p immunoprecipitates were prepared in the absence of NEM, this band was also visible but in a smeared form (Fig. 5A, lane 3), suggesting that degradation intermediates of multiubiquitinated Cbf2p forms could be migrating at this position.

The Cdc34 ubiquitin-conjugating enzyme ubiquitinates Cbf2p in vitro. The experiments described above indicated that

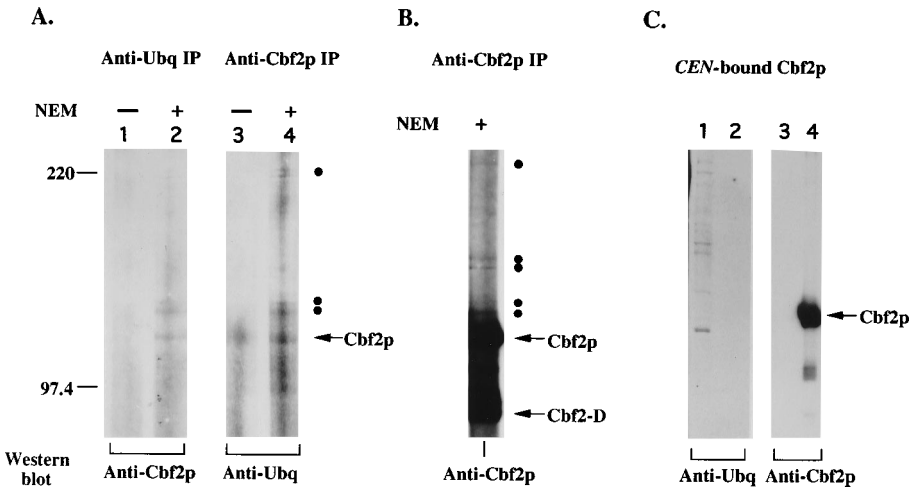

FIG. 5. An antiubiquitin antibody recognizes multiple forms of Cbf2p. (A and B) Antiubiquitin (Anti-Ubq) and anti-Cbf2p immunoprecipitates (IP) were prepared from wild-type yeast cells (strain SEY6210.5) and analyzed by immunoblotting as detailed in Materials and Methods. Before initiation of immunoprecipitation, NEM (10 mM, final concentration) was added to crude nuclear extracts as indicated. Dots, putative ubiquitinated forms of Cbf2p; Cbf2-D, major degradation product of Cbf2p. (C) The CBF3 protein complexes were purified by *CEN* DNA affinity chromatography as described previously (36) and subjected to immunoblot analysis by probing with antiubiquitin (lanes 1 and 2) and anti-Cbf2p (lanes 3 and 4) antibodies. Lanes 1 and 3, 5 μg of affinity column flowthrough fractions; lanes 2 and 4, 0.5 μg of *CEN3* DNA affinity-purified proteins eluted by a standard 500 mM KCl step gradient. The gels were run until the 46-kDa colored marker ran off the bottom. The Cbf2p band is indicated by an arrow; sizes are indicated in kilodaltons.

Cbf2p is ubiquitinated in vivo, and ubiquitin-Cbf2p levels were altered by inactivation of Cdc34p. These data suggest that Cbf2p could be a direct substrate of the *CDC34*-specified ubiquitin-conjugating enzyme. This possibility was examined by examining whether purified Cdc34p in the presence of the appropriate cofactors could catalyze the ubiquitination of Cbf2p in vitro. Covalent attachment of ubiquitins to acceptor proteins, catalyzed by the ubiquitin-conjugating enzymes, requires initial activation of ubiquitin by a ubiquitin-activating enzyme (E1 [38]). Anti-Cbf2p immunoprecipitates prepared from Cbf2p-overproducing yeast cells were used as a source of substrate Cbf2p. Intracellular levels of Cbf2p can be elevated approximately 20-fold, without obvious effects on cell growth, by overexpression from a 2μm-based high-copy-number plasmid containing a 6.5-kb DNA insert bearing the intact *CBF2* gene (plasmid pWJ13-110B) (29). Immunoprecipitated Cbf2p was incubated in the presence of calf thymus E1, purified yeast Cdc34p, ATP, and ^{125}I-labeled ubiquitin. The E1 enzyme has almost the same molecular mass as Cbf2p (~110 kDa) and exhibits a weak autoubiquitination activity (38); therefore, the reaction mixtures were spun briefly after incubation to separate the anti-Cbf2p immunoprecipitates from the soluble E1, Cdc34p, and free ^{125}I-ubiquitin. Before analysis by SDS-PAGE and autoradiography, the pellets were washed under stringent conditions to remove label bound nonspecifically to the immune complex.

Covalent conjugation of ^{125}I-ubiquitin to Cbf2p was observed in the pellet fractions, and only when the reaction mixtures contained both E1 and Cdc34p (Fig. 6B, lane 6). Coomassie blue staining of the same gel revealed Cbf2p in the pellet fractions migrating to a position about 9 kDa below that of the radiolabeled ubiquitin-Cbf2p (Fig. 6A). We interpret this experiment to indicate that Cbf2p is monoubiquitinated in vitro by Cdc34p in a reaction that is dependent on the presence of E1. A minor labeled band in the supernatant fractions, appearing at about the same position as the ubiquitinated Cbf2p, most probably is autoubiquitinated E1, since it appears only in reactions containing E1, in the presence or absence of Cdc34p (Fig. 6B, lanes 2 and 3, arrowhead). Two other smaller labeled proteins are formed in the reactions containing both E1 and Cdc34p (Fig. 6B, lane 3). The identities of these products are unknown, although it is possible that they represent autoubiquitinated forms of Cdc34p (2) or degradation products of Cbf2p-ubiquitin conjugates.

DISCUSSION

In a search for genetic interactions involving yeast kinetochore protein Cbf2p, we isolated *CDC34* as a dosage suppressor of the *ndc10-1* allele. Subsequent biochemical characterization indicated that Cbf2p is modified by ubiquitination in vivo. The *CDC34* ubiquitin-conjugating enzyme apparently is involved directly or indirectly in the formation of Cbf2p-ubiquitin conjugates, since the *cdc34-2* mutation affects the pattern of ubiquitinated Cbf2p forms, and purified Cdc34p in the presence of the appropriate cofactors catalyzes the monoubiquitination of Cbf2p in vitro (Fig. 4 and 6). These data indicate that ubiquitination may play a role in regulation of kinetochore function during the yeast cell cycle.

Among the several ubiquitin-conjugating enzymes of *S. cerevisiae*, Cdc34p is somewhat unique; the protein is essential for the G_1-to-S phase transition and has an unusually long C-terminal tail which includes a 74-residue cell cycle determinant (22, 34, 51). Bacterially expressed yeast Cdc34p is capable of both intermolecular and intramolecular ubiquitin conjugations; the enzyme monoubiquitinates bovine histones H2A and H2B and catalyzes its own ubiquitination in vitro (2, 22). In addition, Cdc34p is a bifunctional enzyme competent in both E3-dependent and -independent conjugation reactions (24). In most cases, monoubiquitin conjugates are formed in an E3-independent way and are not targeted for degradation (12).

To define the role of Cdc34p in the yeast cell cycle, it is crucial to identify physiological substrates of this enzyme. Other studies have shown that *cdc34* mutants are defective in the degradation of the G_1 cyclin Cln2p and the inhibitor of B-type cyclins p40^{SIC1} (15, 45). However, the essential role of Cdc34p may not be confined to the destruction of Cln2p, because *cdc34* mutants fail to enter S phase, whereas hyperacti-

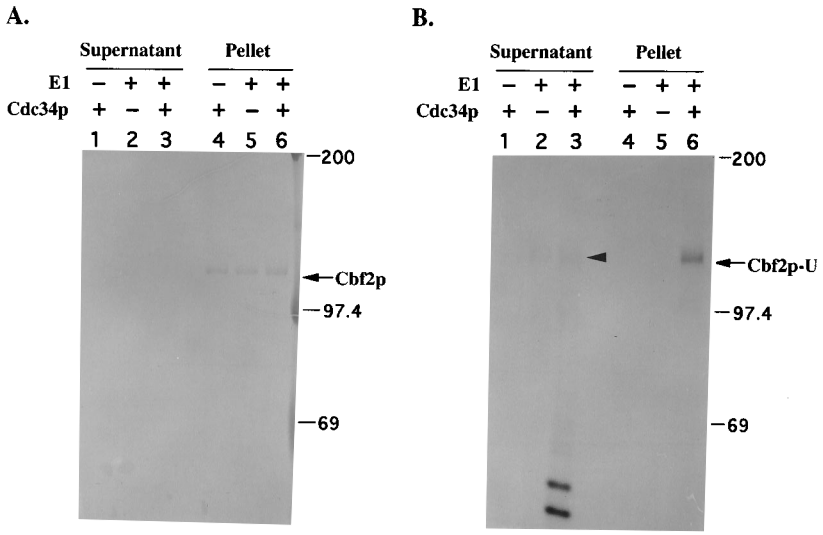

FIG. 6. The *CDC34* ubiquitin-conjugating enzyme (UBC3) ubiquitinates Cbf2p in vitro. Crude nuclear extracts (200 μg) prepared from Cbf2p-overproducing yeast cells (strain SEY6210.5 harboring plasmid pWJ13-110B) were subjected to immunoprecipitation with an anti-Cbf2p antibody. After several washes with buffer A and buffer B, protein A-Sepharose beads containing Cbf2p immune complexes were rinsed with 50 mM Tris (pH 7.5) and resuspended in 30 μl of ubiquitin conjugation assay buffer (see Materials and Methods). While both E1 and Cdc34 enzymes were added to the assay buffer in lanes 3 and 6, E1 and Cdc34p were left out in lanes 1 and 4 and lanes 2 and 5, respectively, as indicated. After 30 min of incubation at 30°C, the pellets (Cbf2p immune complexes) were separated from the supernatants, which contained most of the assay components except Cbf2p. The pellets were washed and analyzed by SDS-PAGE (7.5% polyacrylamide gel) along with the supernatants. The gel was run until the 46-kDa colored marker ran off the bottom, stained with Coomassie blue for 20 min (A), and subjected to autoradiography (B). The X-ray film was exposed for a week at −80°C. Cbf2p-U, Cbf2p-monoubiquitin conjugate. Sizes are indicated in kilodaltons.

vation of G_1 cyclins advances S phase. *SIC1* encodes a potent inhibitor of Clb but not Cln forms of the Cdc28 kinase (41). The $p40^{SIC1}$ protein accumulates as cells exit from mitosis and disappears shortly before S phase by a process that depends on Cdc34p (45). Therefore, destruction of p40 is needed for Clb-Cdc28 kinase activation and entry into S phase. The degradation of p40 also depends on Cdc4p and Cdc53p, two other proteins required in addition to Cdc34p for the G_1-to-S phase transition (45).

It now appears that Cbf2p could be another physiological substrate of the Cdc34 enzyme. Cbf2p has been shown to be a key component of the yeast kinetochore both in vivo and in vitro (23, 30, 36). Our present data suggest that ubiquitin molecules may control Cbf2p function in a cell cycle-dependent way. Interestingly, Cbf2p is associated with the mitotic spindle and the spindle pole bodies in vivo, and some *ndc10* temperature-sensitive alleles (for instance, *ndc10-209*) contain no intact spindles at the nonpermissive temperature, implying that Cbf2p is somehow involved in spindle formation (23, 29, 49). This phenotype is somewhat similar to that of *cdc34* mutant alleles; cells carrying the *cdc34* mutation have defects in the separation of spindle pole bodies and fail to form the mitotic spindle (7, 22). While inactivation of Cdc34p dramatically alters the pattern of ubiquitinated Cbf2p forms, overexpression of *CDC34* causes no significant change in the Cbf2p forms. The *CDC34* ubiquitin-conjugating enzyme may require a specific E3 ubiquitin-protein ligase to carry out the formation of Cbf2p-ubiquitin conjugates in vivo. Further characterization is necessary to determine if Cdc34p directly conjugates ubiquitin molecules to Cbf2p in vivo and how ubiquitination might regulate Cbf2p function.

One of our working models is that ubiquitination may be involved in cell cycle-regulated degradation of Cbf2p in a manner similar to the ubiquitin-mediated proteolysis of *Xenopus* mitotic cyclins (20). Recently, both S- and M-phase B-type cyclins have been shown to be destroyed by the UBC9-dependent proteolytic pathway (46). Proteins conjugated with multiubiquitin chains are targeted for degradation, and Cbf2p seems to be modified by multiubiquitination in vivo; an antiubiquitin antibody recognizes high-molecular-weight forms of Cbf2p (Fig. 5). As shown in the case of the yeast MATα2 repressor, pathways involving multiubiquitination and subsequent degradation can be quite complicated; at least four different ubiquitin-conjugating enzymes target the naturally short-lived α2 protein for degradation by two distinct ubiquitination pathways (11). Thus, if Cbf2p is degraded by the ubiquitin pathway, it is likely that other ubiquitin-conjugating enzymes can participate in marking Cbf2p for proteolysis.

Alternatively, the reversible joining of ubiquitin to Cbf2p could modulate functions without metabolically destabilizing the protein. Ubiquitin forms relatively stable conjugates with histones and actin. A myofibrillar protein termed arthrin is a stable actin-monoubiquitin conjugate present in flight muscles of some insects (1). Moreover, the stable conjugates between H2A (and H2B) and ubiquitin are found preferentially in nucleosomes of transcriptionally active chromatin (6, 37). It has also been reported that all ubiquitinated histones are absent from isolated metaphase chromosomes (55). In our experiments, the Cbf2p-monoubiquitin conjugate formed in vitro was relatively stable, considering that the reaction products containing Cbf2p immune complexes were washed several times before analysis by SDS-PAGE (Fig. 6). On the contrary, the apparent multiubiquitinated forms of Cbf2p were extremely unstable, as evidenced by their rapid disappearance during the course of immunoprecipitation (Fig. 5B). Our data indicate that Cbf2p present in the CBF3 complex purified by *CEN* DNA affinity chromatography is not modified by ubiquitination; the antiubiquitin antibody was unable to supershift the CBF3-*CEN* DNA complex and failed to recognize the 110-kDa subunit of purified CBF3 (Fig. 5C and data not shown). Since Cbf2p is also modified by phosphorylation (31), phosphorylation or dephosphorylation may trigger ubiquitination of Cbf2p. Also, Cbf2p apparently functions both on the kinetochore and in formation of the mitotic spindle; thus, it is likely that ubiq-

uitination is involved in specifying this functional differentiation.

ACKNOWLEDGMENTS

We are grateful to A. Banerjee and V. Chau for providing all reagents used in ubiquitin conjugation assays and for helpful advice on the assay conditions. We thank J. Kilmartin for providing strain JK421 and E. A. Siewert and M. Winey for providing the *ndc10-169* and *ndc10-209* alleles along with information on the phenotypes of these mutants. We thank members of the Carbon and Clarke laboratories for many discussions and technical help, K. Middleton for the generous gift of affinity-purified CBF3 and for numerous discussions, W. Jiang for providing plasmids pWJ13-110B and pWJ316-110B, and E. O'Rourke for preparing media and plates.

H.-J.Y. was supported by a postdoctoral fellowship provided by the Cigarette and Tobacco Surtax Fund of the State of California through the Tobacco-Related Disease Research Program of the University of California (grant 3FT-0096); J.C. is an American Cancer Society Research Professor. This research was supported by NIH research grant CA-11034 from the National Cancer Institute.

REFERENCES

1. **Ball, E., C. C. Karlik, C. J. Beall, D. L. Saville, J. C. Sparrow, B. Bullard, and E. A. Fyrberg.** 1987. Arthrin, a myofibrillar protein of insect flight muscle, is an actin-ubiquitin conjugate. Cell **51:**221–228.
2. **Banerjee, A. L., L. Gregori, Y. Xu, and V. Chau.** 1993. The bacterially expressed yeast *CDC34* gene product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. **268:**5668–5675.
3. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:**17–24.
4. **Bram, R. J., and R. D. Kornberg.** 1987. Isolation of a *Saccharomyces cerevisiae* centromere DNA-binding protein, its human homolog, and its possible role as a transcription factor. Mol. Cell. Biol. **7:**403–409.
5. **Brinkley, B. R.** 1990. Toward a structural and molecular definition of the kinetochore. Cell Motility Cytoskeleton **16:**104–109.
6. **Busch, H., and I. L. Goldknopf.** 1981. Ubiquitin-protein conjugates. Mol. Cell. Biochem. **40:**173–187.
7. **Byers, B., and L. Goetsch.** 1973. Duplication of spindle plaques and integration of the yeast cell cycle. Cold Spring Harbor Symp. Quant. Biol. **38:**123–131.
8. **Cai, M., and R. W. Davis.** 1990. Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell **61:**437–446.
9. **Carbon, J., and L. Clarke.** 1990. Centromere structure and function in budding and fission yeasts. New Biol. **2:**10–19.
10. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28:**145–154.
11. **Chen, P., P. Johnson, T. Sommer, S. Jentsch, and M. Hochstrasser.** 1993. Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MATα2 repressor. Cell **74:**357–369.
12. **Ciechanover, A.** 1994. The ubiquitin-proteasome proteolytic pathway. Cell **79:**13–21.
13. **Cottarel, G., J. H. Shero, P. Hieter, and J. H. Hegemann.** 1989. A 125-base-pair *CEN6* DNA fragment is sufficient for complete meiotic and mitotic centromere functions in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**3342–3349.
14. **Dailey, D., G. L. Schieven, M. Y. Lim, H. Marquardt, T. Gilmore, J. Thorner, and S. Martin.** 1990. Novel yeast protein kinase (*YPK1* gene product) is a 40-kilodalton phosphotyrosyl protein associated with protein-tyrosine kinase activity. Mol. Cell. Biol. **10:**6244–6256.
15. **Deshaies, R. J., V. Chau, and M. Kirschner.** 1995. Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. **14:**303–312.
16. **Doheny, K. F., P. K. Sorger, A. A. Hyman, S. Tugendreich, F. Spencer, and P. Hieter.** 1993. Identification of essential components of the *S. cerevisiae* kinetochore. Cell **73:**761–774.
17. **Elble, R.** 1992. A simple and efficient procedure for transformation of yeasts. BioTechniques **13:**18–20.
18. **Finley, D.** 1992. The yeast ubiquitin system, p. 539–581. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), the molecular and cellular biology of the yeast *Saccharomyces*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Finley, D., and V. Chau.** 1991. Ubiquitination. Annu. Rev. Cell Biol. **7:**25–69.
20. **Glotzer, M., A. W. Murray, and M. W. Kirschner.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature (London) **349:**132–138.
21. **Goebl, M. G., L. Goetsch, and B. Byers.** 1994. The Ubc3 (Cdc34) ubiquitin-conjugating enzyme is ubiquitinated and phosphorylated in vivo. Mol. Cell. Biol. **14:**3022–3029.
22. **Goebl, M. G., J. Yochem, S. Jentch, J. P. McGrath, A. Varshavsky, and B. Byers.** 1988. The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. Science **241:**1331–1335.
23. **Goh, P.-Y., and J. V. Kilmartin.** 1993. *NDC10*: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. J. Cell Biol. **121:**503–512.
24. **Haas, A. L., P. B. Reback, and V. Chau.** 1991. Ubiquitin-conjugation by the yeast *RAD6* and *CDC34* gene products. J. Biol. Chem. **266:**5104–5112.
25. **Hershko, A.** 1991. The ubiquitin pathway for protein degradation. Trends Biochem. **16:**265–268.
26. **Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon.** 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. Nature (London) **359:**533–536.
27. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
28. **Jentsch, S., J. P. McGrath, and A. Varshavsky.** 1987. The yeast DNA repair gene *RAD6* encodes a ubiquitin-conjugating enzyme. Nature (London) **329:**131–134.
29. **Jiang, W., and J. Carbon.** 1993. Molecular analysis of the budding yeast centromere/kinetochore. Cold Spring Harbor Symp. Quant. Biol. **58:**669–675.
30. **Jiang, W., J. Lechner, and J. Carbon.** 1993. Isolation and characterization of a gene (*CBF2*) specifying a protein component of the budding yeast kinetochore. J. Cell Biol. **121:**513–519.
31. **Jiang, W., M.-Y. Lim, H.-J. Yoon, J. Thorner, G. S. Martin, and J. Carbon.** 1995. Overexpression of the yeast MCK1 protein kinase suppresses conditional mutations in centromere-binding protein genes *CBF2* and *CBF5*. Mol. Gen. Genet. **246:**360–366.
32. **Jiang, W., and P. Philippsen.** 1989. Purification of a protein binding to the CDEI subregion of *Saccharomyces cerevisiae* centromere DNA. Mol. Cell. Biol. **9:**5585–5593.
33. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231:**699–704.
34. **Kolman, C. J., J. Toth, and D. K. Gonda.** 1992. Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast CDC34 (UBC3) ubiquitin conjugating (E2) enzyme. EMBO J. **11:**3081–3090.
35. **Lechner, J.** 1994. A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the *Saccharomyces cerevisiae* kinetochore complex, Cbf3. EMBO J. **13:**5203–5211.
36. **Lechner, J., and J. Carbon.** 1991. A 240 kDa multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. Cell **64:**717–725.
37. **Levinger, L., and A. Varshavsky.** 1982. Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the Drosophila genome. Cell **28:**375–385.
38. **McGrath, J. P., S. Jentsch, and A. Varshavsky.** 1991. *UBA1*: an essential yeast gene encoding ubiquitin-activating enzyme. EMBO J. **10:**227–237.
39. **McGrew, J., B. Diehl, and M. Fitzgerald-Hayes.** 1986. Single base-pair mutations in centromere element III cause aberrant chromosome segregation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**530–538.
40. **Meluh, P., and M. D. Rose.** 1990. *KAR3*, a kinesin-related gene required for yeast nuclear fusion. Cell **60:**1029–1041.
41. **Mendenhall, M. D. M.** 1993. An inhibitor of $p34^{CDC28}$ protein kinase activity from *Saccharomyces cerevisiae*. Science **259:**216–219.
42. **Middleton, K., and J. Carbon.** 1994. *KAR3*-encoded kinesin is a minus-end-directed motor that functions with centromere binding proteins (CBF3) on an in vitro yeast kinetochore. Proc. Natl. Acad. Sci. USA **91:**7212–7216.
43. **Neigeborn, L., and A. P. Mitchell.** 1991. The yeast *MCK1* gene encodes a protein kinase homolog that activates early meiotic gene expression. Genes Dev. **5:**533–548.
44. **Ng, R., and J. Carbon.** 1987. Mutational and in vitro protein-binding studies on centromere DNA from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**4522–4534.
45. **Schwob, E., T. Böhm, M. D. Mendenhall, and K. Nasmyth.** 1994. The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1 to S transition in S. cerevisiae. Cell **79:**233–244.
46. **Seufert, W., B. Futcher, and S. Jentsch.** 1995. Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. Nature (London) **373:**78–81.
47. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Shero, J. H., and P. Hieter.** 1991. A suppressor of a centromere DNA mutation encodes a putative protein kinase (*MCK1*). Genes Dev. **5:**549–560.
49. **Siewert, E. A., and M. Winey (University of Colorado).** 1994. Personal communication.
50. **Sikorski, R. S., and P. Hieter.** 1992. A system of shuttle vectors and yeast

host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
51. **Silver, E. T., T. J. Gwozd, C. Ptak, M. Goebl, and M. J. Ellison.** 1992. A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2): implications for the structure, function, and evolution of the E2s. EMBO J. **11:**3091–3098.
52. **Sorger, P. K., F. F. Severin, and A. A. Hyman.** 1994. Factors required for the binding of reassembled yeast kinetochores to microtubules in vitro. J. Cell Biol. **127:**995–1008.
53. **Tyers, M., G. Tokiwa, R. Nash, and B. Futcher.** 1992. The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. **11:**1773–1784.
54. **Varshavsky, A.** 1992. The N-end rule. Cell **69:**725–735.
55. **Wu, R. S., K. W. Kohn, and W. M. Bonner.** 1981. Metabolism of ubiquitinated histones. J. Biol. Chem. **256:**5916–5920.