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Identification of a Positive Regulator of the Cell Cycle Ubiquitin-Conjugating Enzyme Cdc34 (Ubc3)

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The Cdc34 (Ubc3) ubiquitin-conjugating enzyme from *Saccharomyces cerevisiae* plays an essential role in the progression of cells from the G_1 to S phase of the cell division cycle. Using a high-copy suppression strategy, we have identified a yeast gene (*UBS1*) whose elevated expression suppresses the conditional cell cycle defects associated with *cdc34* mutations. The *UBS1* gene encodes a 32.2-kDa protein of previously unknown function and is identical in sequence to a genomic open reading frame on chromosome II (GenBank accession number Z36034). Several lines of evidence described here indicate that Ubs1 functions as a general positive regulator of Cdc34 activity. First, overexpression of *UBS1* suppresses not only the cell proliferation and morphological defects associated with *cdc34* mutants but also the inability of *cdc34* mutant cells to degrade the general amino acid biosynthesis transcriptional regulator, Gcn4. Second, deletion of the *UBS1* gene profoundly accentuates the cell cycle defect when placed in combination with a *cdc34* temperature-sensitive allele. Finally, a comparison of the Ubs1 and Cdc34 polypeptide sequences reveals two noncontiguous regions of similarity, which, when projected onto the three-dimensional structure of a ubiquitin-conjugating enzyme, define a single region situated on its surface. While *cdc34* mutations corresponding to substitutions outside this region are suppressed by *UBS1* overexpression, Ubs1 fails to suppress amino acid substitutions made within this region. Taken together with other findings, the allele specificity exhibited by *UBS1* expression suggests that Ubs1 regulates Cdc34 by interaction or modification.

The timing of events that guide cells through the various stages of cell growth and division necessarily depends on the synthesis or activation of proteins that specify one cell cycle stage coupled with the inactivation or degradation of proteins that specified the previous cell cycle stage. In recent years, it has become apparent that the ubiquitin (Ub)-dependent proteolytic system plays an important role in regulating the steady-state levels of a number of key cell cycle regulators including the mitotic cyclins (16, 37), the p53 tumor suppressor (34, 35), and the c-Jun transactivator (44). In the yeast *Saccharomyces cerevisiae*, this system has been shown to be functionally important in at least three of the five stages that define the cell cycle: G_0 to G_1, G_1 to S, and G_2 to M. The requirement for Ub-dependent proteolysis at each of these stages has been inferred from mutational studies in *S. cerevisiae* that demonstrate the dependence of each stage on a different member of a class of proteins referred to as the Ub-conjugating enzymes or E2s. These enzymes mediate the transfer of Ub from the Ub-activating enzyme E1 to their protein targets, thereby tagging them for elimination (14, 20, 22, 24). Ubc1, for example, mediates the G_0-to-G_1 transition that accompanies spore germination (38). Cdc34 (Ubc3) is critically involved in the G_1-to-S transition (6), whereas a newly discovered Ub-conjugating enzyme, Ubc9, plays an essential role at mitosis (37). The present study deals with the G_1-specific Ub-conjugating enzyme, Cdc34.

The *CDC34* gene was originally identified as a conditional cell cycle mutation that arrested cells after the Start regulatory step but before S phase (6). Cells arrested at the Cdc34-dependent step are therefore unable to proceed with nuclear DNA replication but remain competent to duplicate the spindle pole body and produce buds periodically (6). The Cdc34 protein consists of the moderately conserved catalytic domain that is characteristic of all Ub-conjugating enzymes and a C-terminal extension or tail that is unique to Cdc34 (18). The tail of Cdc34 is essential for its cell cycle function and can confer its functional properties on the DNA repair Ub-conjugating enzyme Rad6 when appended to its C terminus (26, 41). Genetic evidence suggests that the tail mediates the interaction of Cdc34 with itself and other E2s and that this interaction is necessary for its function (41). This idea has been recently strengthened by the observation that both the cell cycle determinant and the ability of Cdc34 to interact with itself in vitro colocalize to a 29-residue segment of the 125-residue tail (33).

On the basis of published evidence, several candidates for the target of Cdc34 ubiquitination have been proposed. Tyers et al. have suggested that the histone H1 kinase activity that is associated with the G_1 cyclin Cln3 may be a substrate of Cdc34-dependent proteolysis on the basis of its elevated levels in *cdc34* mutants (45). In related work, it has been found that Cln3 is stabilized by a loss of Cdc34 function (46). Deshaies et al. have also provided evidence that degradation of another cyclin, Cln2, requires Cdc34 function (8). McKinney et al. have suggested that the Start regulator, Far1, may be a target of Cdc34 on the basis of the increased levels of phosphorylated Far1 in cells defective for Cdc34 (28). In addition, Schwob et al. have recently provided strong evidence that the Cdc28 inhibitor p40-Sic1 is the Cdc34 substrate responsible for S-phase inhibition in *cdc34* mutant cells (36). Finally, Kornitzer et al. (27) have recently demonstrated that the rapid turnover of Gcn4, a global regulator of amino acid biosynthesis, depends upon Cdc34. Since Gcn4 function bears no obvious relation-

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ship with cell cycle regulation, these results indicate that the Cdc34 protein is a multifunctional regulator.

At present, little is known about whether Cdc34 is subject to the same forms of posttranslational regulation that are characteristic of other cell cycle regulators; however, the recent observations that Cdc34 is both phosphorylated (17) and ubiquitinated (4, 17) suggest that posttranslational regulation is likely. In this work, we describe a general positive regulator of Cdc34 which we have called Ubs1 (for Ub-conjugating enzyme suppressor 1).

MATERIALS AND METHODS

Plasmids. Four plasmids were used in the physical and functional characterization of the *UBS1* gene. The sequence of the *UBS1* gene was determined from a plasmid (p24A1) that contained a 1.77-kb *Sau*3A fragment cloned into the *Bam*HI site of the high-copy yeast plasmid YEP352 (21). This fragment was originally derived from a larger genomic fragment contained on the high-copy PMA3A yeast plasmid (described in reference 9) that was isolated in the genetic screen described below. In addition to the coding sequence of *UBS1*, the *Sau*3A fragment also contained 630 bp to the 5′ side of the Met-1 codon and 285 bp to the 3′ side of the termination codon. The fragment is oriented in YEP352 such that the Met-1 codon lies proximal to the *Hin*dIII site of the multiple cloning region.

The *UBS1* allele was deleted in selected yeast strains with the integration plasmid pUBS1Δ. This plasmid was constructed by replacing the 1,044-bp *Bgl*II-*Hpa*I fragment (containing codons 1 to 219 of *UBS1*) of p24A1 (Fig. 1) with the *Bgl*II-*Cla*I fragment (containing the *TRP1* gene) derived from plasmid YRP7 (42). To facilitate the ligation of the *Cla*I end of the fragment to the *Hpa*I end of the plasmid, the *Cla*I site was filled in prior to ligation. The replacement of the *UBS1* gene with the *TRP1* gene results in the deletion of about 80% of the *UBS1* coding sequence.

For expression experiments involving *Ubs1*, an expression plasmid (SP2) in which the *UBS1* coding sequence was placed under the control of the copper-inducible *CUP1* promoter was constructed. This high-copy plasmid contains the *TRP1* selectable marker and is identical to the Ub expression plasmid YEP96, except that the *UBS1* coding sequence replaces the Ub-encoding sequence (12). In addition, a Met codon and a Ser codon were placed in front of the *UBS1* gene Met-1 codon, introducing an *Sst*I site within the *UBS1* coding sequence.

The high-copy *CDC34-TRP1* expression plasmid used in this study has been previously described (41) and is identical to the *UBS1* expression plasmid SP2 except that the coding sequence of *CDC34* replaced the coding sequence of *UBS1*. The low-copy *cdc34-1* expression plasmid contains the temperature-sensitive (ts) *cdc34-1* allele expressed from the *CUP1* promoter carried on the low-copy *URA3*-based plasmid PRS316 (40). This expression plasmid was constructed in two steps. First, the *cdc34-2* allele (obtained from strain mGG16 by PCR methods [11]) was used to replace the wild-type *CDC34* gene in the *CDC34-TRP1* plasmid described above (41). Second, the *CUP1* promoter, *cdc34-1* allele, and the *CYC1* terminator were removed from the *TRP1* high-copy plasmid by *Bam*HI-*Cla*I digestion and ligated into the *Bam*HI-*Cla*I sites of the centromere-based plasmid PRS316 (40). The low-copy *cdc34-2* expression plasmid was prepared as described for the *cdc34-1* expression plasmid except that the yeast strain YL10 was used as a source of the *cdc34-2* allele (32). The low-copy *cdc34-3* expression plasmid is also based on the PRS316 plasmid used to construct the low-copy *cdc34-1* and *cdc34-2* plasmids. The *cdc34-3* allele, however, unlike the *cdc34-1* and *cdc34-2* alleles, was not obtained from mutant chromosomal genes but rather was produced by site-directed mutagenesis of a wild-type *CDC34* gene cassette. Specifically, the *cdc34-3* allele was prepared with a mutagenic oligonucleotide (ggatggtaga tagccggcgt aaatcgaaac tgtggtggtt tattgggaaa gtcttccgga aattccattt gagc), the wild-type *CDC34* gene, and the vectors and conditions described for the Altered States in vitro mutagenesis system (Promega). Successful conversion of the wild-type *CDC34* gene to the *cdc34-3* mutant allele was confirmed by sequence analysis. The three ts *cdc34* mutations were caused by Pro-71-to-Ser substitution associated with the *cdc34-1* mutation (11); the Gly-58-to-Arg mutation associated with the *cdc34-2* mutation (32); and the Arg-65-to-Glu, Phe-72-to-Asn, and Ser-73-to-Lys substitutions associated with the *cdc34-3* mutation.

The pES13 plasmid, which was used as a negative control in experiments with expression plasmids containing the *TRP1* selectable marker, is identical to YEP96 except that the coding sequence of the Ub gene was deleted between the unique *Bgl*II and *Sal*I sites. The YEP352 plasmid (21) was used as a negative control in examining the effect of *URA3*-based expression vectors.

The stability of the Gcn4 protein was assessed in *S. cerevisiae* by a fusion of the *GCN4* and *lacZ* coding sequences under control of the galactose-inducible *GAL1* promoter. *GCN4* was placed under control of the *GAL1* promoter by cloning the *Hin*dIII-*Eco*RI fragment of YCp88-*GCN4* (23) into vector pDAD1 (kindly provided by D. Miller and D. Pellman), to generate pGAL-GCN4. For the *GCN4-lacZ* fusion, PCR methods were used (2) to clone a DNA fragment carrying the complete *GCN4* coding sequence under the *GAL1,10* promoter from plasmid pGAL-GCN4 into vector YEp357R (30), generating KB64. The details that relate to construction of all these plasmids are available on request.

Yeast strains. Two *cdc34* mutant strains were used for the cloning and subsequent functional characterization of *UBS1*. The ts *cdc34-2* mutant strain JP34G2 (*MAT***a** *ade2-1 his3*Δ *leu2*Δ*1 trp1 ura3-52 cdc34-2*) was constructed from a cross of the *cdc34-2* mutant strain YL10 (*MAT***a** *leu2*Δ*1 his3*Δ *trp1*Δ*63 ura3-53 cdc34-2*) and the wild-type strain KMY15 (*MAT***a** *ade2-1 his3-832 trp1-289 ura3-52*). YL10 was obtained from M. Goebl (University of Indiana), and KMY15 was obtained from K. Madura (California Institute of Technology). The *CDC34* disruption strain YES71 was created from YL10 by disrupting the *cdc34-2* allele with the *Apa*I-*Eco*RI fragment from pGEM34H/S as previously described (18). Viability of YES71 was maintained by the *CDC34-TRP1* plasmid (described above) or a low-copy plasmid containing the *cdc34-1*, *cdc34-2*, or *cdc34-3* allele (described above).

cdc34 mutant and wild-type strains that carried a deletion of the *UBS1* allele were constructed by integrating the *Bgl*II-*Hpa*I fragment carrying the *TRP1* gene from the pUBS1Δ plasmid (described above) into the *UBS1* allele of JP34G2 (*cdc34-2*) and KMY15 (wild-type *CDC34*). *TRP1* prototrophs from the correct integration of the *UBS1*Δ*-TRP1* fragment at the *UBS1* locus were selected on the basis of two criteria: (i) the presence of a unique *Xba*I site at the *UBS1* locus that accompanies the introduction of the *TRP1* marker and (ii) the loss of the *UBS1* mRNA. In the first method, primers homologous to the 3′ end of the *TYR1* gene (ccgctgatac acaagctgtg acacatgctg) and 5′ noncoding flank of the *UBS1* gene (gaactaaaaa tgttacccat gttgatc) were used in combination with the PCR to obtain 1.2 kb of DNA from the chromosomal *UBS1* locus. Successful replacement of the *UBS1* gene by the *TRP1* gene results in the insertion of a unique *Xba*I site (from the *TRP1* gene) into the center of this DNA fragment. Therefore, sensitivity of the above-mentioned PCR product to *Xba*I digestion indicates the replacement of the *UBS1* gene by the *TRP1* gene.

In the second method, the levels of cellular *UBS1* mRNA were measured by reverse transcription followed by the specific amplification of a segment of *UBS1* cDNA by PCR. In the first step, total RNA was isolated by hot phenol extraction (2) from cells suspected to contain a disruption of the *UBS1* gene. With a cDNA primer specific for the *UBS1* transcript (gtggctttga gcattgtaag), a cDNA strand was prepared from the RNA template as described previously (31). In the second step, PCR was performed with these cDNA products and primers specific for the *UBS1* gene (*UBS1*-specific primer above and gtggtgtacgagcc caggacttct). The absence of the expected amplified 437-bp fragment relative to wild-type cells indicated the loss of *UBS1* gene function. Yeast strains fulfilling both of the criteria (described above) have been designated JP34G2-*UBS1*Δ and KMY15-*UBS1*Δ.

Gcn4 stability was measured in the wild-type yeast strain KY130 and the isogenic *cdc34-2* mutant strain KY203 (27) under conditions described below.

Isolation of high-copy suppressors of the *cdc34-2* mutation. To identify high-copy suppressors of the *cdc34-2* mutation, cells from strain JP34G2 were transformed with a genomic library on the high-copy vector PMA3A (constructed by Mick Tuite, University of Kent). This library contained 10- to 15-kb *Sau*3A fragments of yeast genomic DNA ligated into the *Bam*HI site of the *LEU2*-based plasmid PMA3A (described in reference 9). Suppressor plasmids were identified by their ability to allow *cdc34-2* mutant cells to form colonies on yeast extract-peptone-dextrose (YEPD) plates (39) at a restrictive temperature of 32°C. This temperature was found to confer slow growth on *cdc34* mutants and was chosen so as not to exclude weak suppressors from the selection. Plasmids were isolated from temperature-resistant yeast cells and transformed into *Escherichia coli* cells. Plasmids propagated in *E. coli* were amplified, purified, and reintroduced into *cdc34-2* mutant cells in order to confirm their suppressor ability. Plasmids still competent to suppress the *cdc34-2* mutation after this second selection were grouped into families on the basis of restriction and Southern analysis (2). Subcloning of each suppressor gene was performed by partial *Sau*3A digestion of a single plasmid isolate from each family (2) followed by ligation of these fragments into the *Bam*HI site of the shuttle vector YEP352 (21). Sublibraries were then retransformed into the *cdc34-2* mutant strain to identify plasmids with the smallest DNA inserts still able to suppress the *cdc34-2* mutation. Selected plasmids were reisolated from *S. cerevisiae* and sequenced. Sequencing of double-stranded plasmid DNA by the dideoxy chain-termination method was performed on an Applied Biosystems automated Sequenator operated by the University of Alberta DNA Sequencing and Synthesis Facility. The *UBS1* gene was sequenced in both strands and searched against the GenBank DNA and protein database. A perfect nucleotide match was found against an unidentified open reading frame (ORF) (SBR165W) that was identified during the sequencing of chromosome II (13).

Effect of *UBS1* overexpression on the relative abundance of the Cdc34-2 protein. The effect of *UBS1* overexpression on the abundance of the Cdc34-2 protein was examined in *cdc34-2* mutant cells at both the permissive and nonpermissive temperatures. Briefly, log-phase JP34G2 cells, containing either the *UBS1* overexpression plasmid (p24A1) or a negative control plasmid (YEP352), were shifted from the permissive temperature of 25°C to the nonpermissive temperature of 34°C. Cells were harvested before the temperature shift and after 3 h at the nonpermissive temperature. Conditions used for the preparation of protein extracts, gel electrophoresis, and Western blotting (immunoblotting) have been previously described (17). The Cdc34-2 protein was localized by probing an immunoblot with an anti-Cdc34 antibody (17) and visualized by using

the enhanced chemiluminescence Western detection system (Amersham). Total protein determinations of cell lysates were performed by the Bio-Rad assay, and 20 μg of protein was loaded into each gel lane. Comparison of band intensities was performed with a Color OneScanner (Macintosh) and MacBas version 2.0 software with an autoradiogram falling within the linear range of exposure.

Suppression analysis of the *UBS1* gene in *cdc34* mutant strains. The ability of the *UBS1* gene to suppress ts *cdc34* mutations was assessed both in the *cdc34-2* strain JP34G2 and in strain YES71 (with *cdc34* deleted) carrying a plasmid with either the *cdc34-1*, *cdc34-2*, or *cdc34-3* ts allele. When the JP34G2 strain was used, the *UBS1* gene was expressed from either the *TRP1-UBS1* plasmid SP2 or the *URA3-UBS1* plasmid p24A1. Overexpression of the *UBS1* gene in the YES71 strain was assessed by using the SP2 plasmid. The important difference between plasmids SP2 and p24A1 is that plasmid p24A1 expresses the *UBS1* gene from the native *UBS1* promoter, while plasmid SP2 expresses the *UBS1* gene from the *CUP1* promoter.

The ability of *UBS1* overexpression to suppress the *cdc34* proliferation defect was assessed by colony formation on YEPD plates or growth in broth (39) at specific nonpermissive temperatures. The nonpermissive temperature for either the *cdc34-2* mutant strain or the deletion strain (YES71) expressing one of three *cdc34* ts alleles was chosen as the lowest restrictive temperature at which mutant cells were still unable to form visible colonies after 3 days of incubation. By this criterion, the nonpermissive temperature for the *cdc34-2* mutant strain was 34°C, while the deletion strain had a nonpermissive temperature of 37°C when expressing the *cdc34-1* and *cdc34-2* alleles and 38°C when expressing the *cdc34-3* allele. At 38°C, *cdc34-3* mutant cells are still able to form colonies but grow more slowly than wild-type cells and display the *cdc34* mutant phenotype.

Cultures of *cdc34* mutant cells displaying a multibudded or aberrant cell cycle morphology were prepared by incubating JP34G2 cells in a temperature-controlled water bath in either YEPD or fully supplemented SC broth (39). Cells were fixed by removing aliquots from these liquid cultures and adding them to a ninefold excess of 0.08% saline and 3% formaldehyde (19). After cells were fixed, loosely attached cells were separated by sonication with a Vibra-Cell sonicator (Sonics and Materials, Danbury, Conn.) at 50% power for 10 s. The frequency of cells displaying a particular cell morphology was determined by microscopic examination of at least 100 cells for each time point. Analysis of Gcn4 stability was determined as described previously (27).

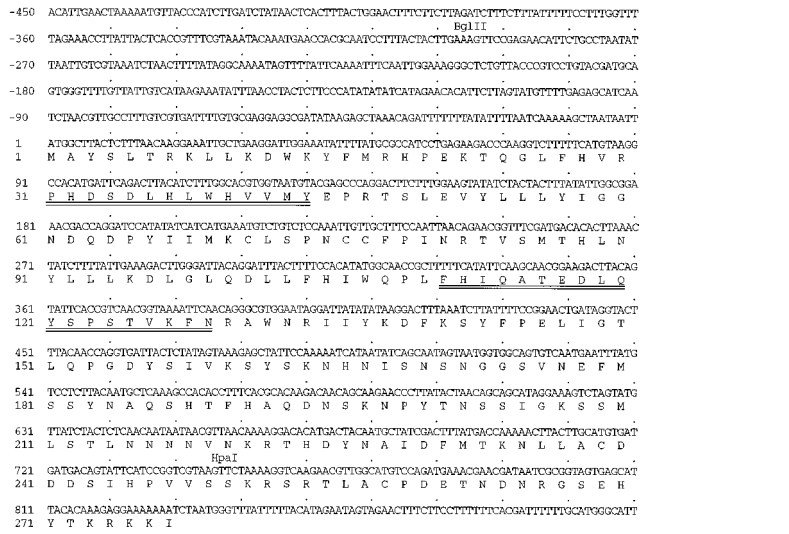

FIG. 1. Sequence of the *UBS1* gene. The *UBS1* ORF begins at position 1 and ends with a stop codon at position 832. The predicted Ubs1 peptide sequence is represented by the single-letter amino acid code immediately below the corresponding codon. The two regions of the Ubs1 peptide sequence showing similarity to the Cdc34 and wheat Ubc1 Ub-conjugating enzymes (Fig. 7) have been underscored with a double line. The sequence presented here is identical to sequence YBR165w, an unidentified ORF submitted to the GenBank database (13). The *Hpa*I and *Bgl*II restriction sites used in the construction of pUBS1Δ (Materials and Methods) have been indicated by the name of the restriction enzyme directly below the recognition sequence.

RESULTS

Isolation of the *UBS1* gene as a high-copy suppressor of the *cdc34-2* mutation. In this study, a genetic screen was employed to identify genes that when overexpressed could increase Cdc34 activity. We reasoned that suppressors able to increase Cdc34 activity could function in at least one of three ways. First, overexpression of a protein that normally interacts with Cdc34 might suppress the *cdc34-2* mutation by stabilizing the temperature-labile Cdc34-2 protein. Second, overexpression of proteins that normally prevent turnover or inactivation of the Cdc34-2 protein could also suppress the *cdc34-2* mutation. Finally, overexpression of a protein that down-regulated the levels of the putative Cdc34 cell cycle target might also be expected to suppress the defect associated with the *cdc34-2* mutation. With these mechanisms in mind, high-copy suppressors of the *cdc34-2* mutation were identified by their ability to enhance mutant cell growth at the normally restrictive temperature of 32°C. From approximately 80,000 transformed cells, 28 colonies were selected. On the basis of Southern analysis, the library plasmids that were isolated from those colonies defined three plasmid families. The first plasmid family had 16 members and was shown to contain the wild-type *CDC34* gene. The second family contained five members and by sequence analysis was found to contain the poly-Ub gene, *UBI4*. The suppression of the *cdc34-2* mutation by the *UBI4* gene is the subject of another report (32). The third plasmid family, with seven independent isolates, contained a novel gene which is the subject of this paper.

A representative plasmid from this third family, pI2, was subcloned by partial *Sau*3A digestion to generate a series of plasmids with different-size DNA fragments (Materials and Methods). From this series, a plasmid (p24A1) that contained the smallest-sized DNA fragment still able to suppress the *cdc34-2* mutation was obtained. This plasmid was used in some expression studies and as a template for sequencing. The sequence of the *Sau*3A fragment contained on plasmid p24A1 revealed one major ORF of 831 bases positioned in the center of the 1.77-kb genomic fragment that encodes a 32.2-kDa polypeptide (Fig. 1). We have named this ORF *UBS1* for Ub-conjugating enzyme suppressor 1. By using the Blast protein and DNA database searching service (1), *UBS1* was found to be identical to sequence YBR165w, an ORF of unknown function identified during the sequencing of chromosome II (13).

The *UBS1* promoter region was searched against both transcription factor (15) and eukaryotic promoter (Blast program [1]) databases. Outside of putative TATA boxes and cap-binding sites within 140 bases of the start codon, no previously described transcription factor recognition sequences were found in the *UBS1* promoter region.

Ubs1 is a positive effector of Cdc34's cell cycle activity. To establish that the *UBS1* gene on plasmid p24A1 encoded the *cdc34-2* suppressor, an expression plasmid that placed only the *UBS1* coding sequence under the regulation of the *CUP1* copper-inducible promoter was created (Materials and Methods). *cdc34-2* mutant cells (JP34G2) transformed with this expression plasmid (SP2) and plated at the nonpermissive temperature of 34°C were able to form visible colonies after 3 days of incubation (Fig. 2). In contrast, *cdc34-2* mutant cells not overexpressing the *UBS1* gene were unable to form visible colonies under these conditions. In subsequent experiments, the *UBS1* gene was found to suppress the *cdc34-2* mutation as effectively as the entire original genomic fragment (data not shown). Therefore, the *UBS1* gene encodes a polypeptide able to suppress the *cdc34-2* mutation.

In JP34G2 cells (*cdc34-2*), increased expression of the *UBS1* gene by induction of the copper promoter (*CUP1*) with $CuSO_4$ did not increase the suppressor ability of the expression plasmid with respect to the original genomic plasmid p24A1 (data not shown). This observation indicates that the optimal level of expression of the *UBS1* gene necessary to suppress the *cdc34-2*

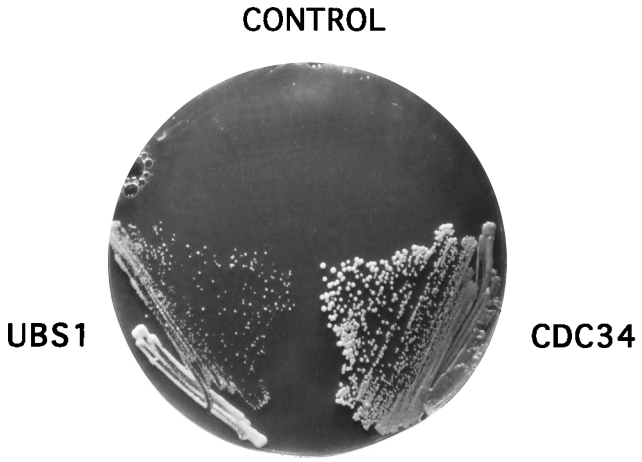

FIG. 2. Overexpression of the *UBS1* gene suppresses the cell proliferation defect associated with the *cdc34-2* mutation. JP34G2 cells (*cdc34-2*) transformed with either the *UBS1* or *CDC34* expression plasmid or the negative control plasmid PES13 were streaked on YEPD plates and incubated for 3 days at 34°C.

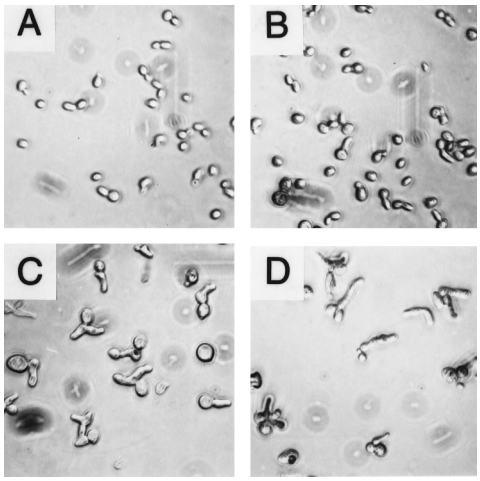

FIG. 4. *cdc34-2* mutants with *UBS1* deleted display an accentuated cell cycle defect. (A, B, and C) Morphology of *cdc34-2* mutant cells that were shifted from the permissive (25°C) to the nonpermissive (34°C) temperature for 0, 3, and 6 h, respectively. (D) Morphology of the *cdc34-2 ubs1*Δ double mutant incubated at 25°C.

mutation can be supplied either by increased dosage of the genomic sequence or by the already high level of basal transcription from the *CUP1* promoter (10).

Other cell cycle phenotypes associated with the *cdc34* mutation are also suppressed by the *UBS1* gene. One *cdc34* mutant phenotype originally associated with the cell proliferation defect is the formation of cells with multiple buds in cultures incubated at restrictive temperatures (6). As shown in Fig. 3, overexpression of the *UBS1* gene reduces by fourfold the frequency of multibudded cells in *cdc34-2* cultures (strain JP34G2) incubated at the restrictive temperature of 34°C. This reduction in the frequency of multibudded cells is seen whether the *UBS1* gene is expressed from its native promoter or the CUP1 promoter (data not shown). These results demonstrate that overexpression of the *UBS1* gene suppresses both the proliferation defect associated with the *cdc34-2* mutation and its associated mutant cell phenotype.

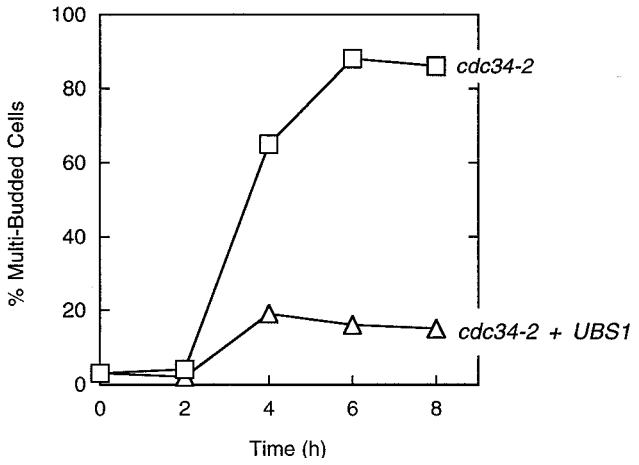

FIG. 3. Overexpression of the *UBS1* gene reduces the frequency of multibudded cells in *cdc34-2* mutant cell cultures incubated at the nonpermissive temperature. Log-phase cultures of JP34G2 cells (*cdc34-2*) with (*cdc34-2 + UBS1*) or without (*cdc34-2*) the *UBS1* plasmid were shifted from 25°C to the nonpermissive temperature of 34°C. Cells were then collected from log-phase cultures at 2-h intervals and fixed, and the frequency of multibudded cells was determined by microscopic examination.

The results described above show that overexpression of the *UBS1* gene can partially suppress cellular defects associated with a loss of Cdc34 function. We next examined the effect on the cell cycle produced by deleting the *UBS1* gene from either wild-type cells or *cdc34-2* mutant cells. Deletion of the *UBS1* gene in a wild-type strain (KMY15) had no effect on viability, and cells were morphologically indistinguishable from wild-type cells with an intact *UBS1* gene. Furthermore, these cells displayed none of the other obvious phenotypes normally associated with defects in Ub conjugation, including temperature and canavanine sensitivity (38), sensitivity to UV light (25), or a defect in either sporulation or germination (25, 38). Therefore, the *UBS1* gene appears to be nonessential when present in a wild-type *CDC34* background. In sharp contrast, however, *cdc34-2* mutant cells that had lost Ubs1 function displayed extremely poor cell viability and slow growth at room temperature, 9°C lower than the normal nonpermissive temperature for *cdc34-2* mutant cells. Microscopic examination revealed that a large portion of these cells express a multibudded mutant phenotype normally seen in *cdc34-2* mutant cells after incubation at the nonpermissive temperature (Fig. 4D). Since *cdc34-2* mutant cells do not normally display these mutant phenotypes at the permissive temperature of 25°C, these results indicate that a loss of Ubs1 function causes a significant decrease in Cdc34-2 activity. As expected, the enhanced *cdc34* mutant phenotype caused by deletion of the *UBS1* gene can be reversed by introduction of the *UBS1* overexpression plasmid (data not shown).

Although the *UBS1* gene is not essential for wild-type cells under the range of conditions tested, the suppression of *cdc34-2* mutants by elevated levels of the Ubs1 protein coupled with the heightened severity of the Cdc34-2 phenotype upon loss of Ubs1 strongly correlates the Ubs1 protein with a normal role in Cdc34 function.

***UBS1* overexpression does not alter the relative abundance of the Cdc34-2 protein.** In earlier studies, we found that either increased gene dosage or transcription of ts *cdc34* alleles resulted in the suppression of the ts defect normally associated with that particular allele (data not shown). This loss of temperature sensitivity is likely caused by an increase in the abundance of the partially functional Cdc34 mutant protein which

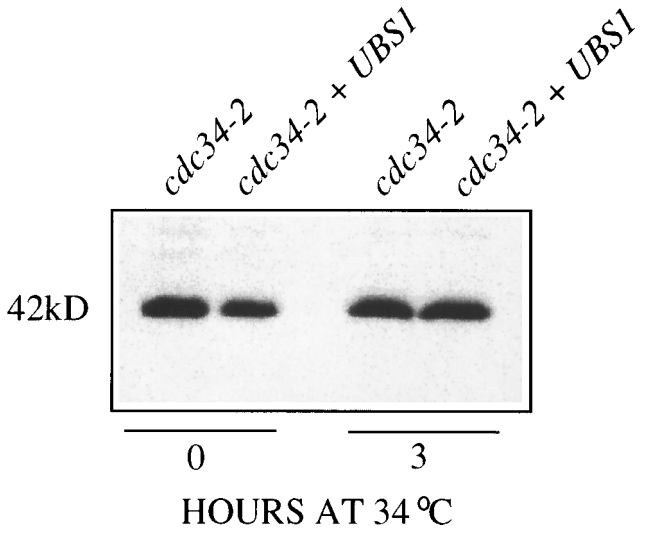

FIG. 5. Overexpression of the *UBS1* gene does not increase the relative abundance of the Cdc34-2 protein. Log-phase *cdc34-2* mutant cells with or without the *UBS1* overexpression plasmid were harvested at 25°C (0 h) or after 3 h at 34°C. The Cdc34-2 protein was visualized by Western analysis with an anti-Cdc34 antibody as described previously (17) (see also Materials and Methods).

in turn leads to increased cellular levels of Cdc34 activity. To determine if *UBS1* suppression operates by a mechanism that increases the level of Cdc34-2, we directly examined the abundance of the Cdc34-2 protein in cells overexpressing the *UBS1* gene. In these experiments, log-phase cultures of *cdc34-2* mutant cells, with or without the *UBS1* overexpression plasmid, were harvested either at the permissive temperature of 25°C or after 3 h at the nonpermissive temperature of 34°C. Examination of immunoblots prepared with total cellular protein and probed with an anti-Cdc34 antibody showed that the level of the Cdc34-2 protein in cells overexpressing the *UBS1* gene was indistinguishable from the level of the Cdc34-2 protein in the absence of *UBS1* overexpression when incubated at the nonpermissive temperature (Fig. 5). Therefore, the mechanism by which Ubs1 suppresses the *cdc34-2* mutation does not involve an increase in the abundance of the Cdc34-2 protein by either a transcriptional, translational, or posttranslational process.

***UBS1* overexpression can partially restore the ability of *cdc34* mutant cells to degrade Gcn4.** A recent report has shown that Cdc34 is involved in the ubiquitination and turnover of the general amino acid control protein Gcn4 (27). Since mutations that influence Gcn4 activity exhibit none of the cell cycle defects associated with *cdc34* mutations, it is clear that Gcn4 represents a target of Cdc34 that is functionally unrelated to the cell cycle phenotypes associated with *cdc34* mutants. To test whether *UBS1* overexpression can influence Gcn4 turnover, we examined the half-life of a Gcn4–β-galactosidase fusion protein (27) expressed in wild-type or *cdc34-2* mutant cells in the presence or absence of the high-copy *UBS1* plasmid. The results of this experiment are shown in Fig. 6. In wild-type cells Gcn4–β-galactosidase was degraded at 34°C with an estimated half-life of 3 min. In contrast, the half-life of Gcn4–β-galactosidase in *cdc34-2* mutant cells increased 13-fold to 40 min. Introduction of the *UBS1* plasmid into these cells, however, resulted in a relative threefold stabilization of Gcn4–β-galactosidase, yielding a half-life of approximately 14 min. Thus, *UBS1* overexpression partially suppresses the Gcn4 turnover defect associated with loss of Cdc34 function and is therefore not restricted to an exclusive role in the cell cycle.

The Ubs1 protein exhibits similarity to a defined region on the surface of Ub-conjugating enzymes. A comparison of the Ubs1 polypeptide sequence with the SwissProt database revealed similarity to the previously characterized wheat Ub-conjugating enzyme Ubc1 (accession number P25856 [43]). Upon visual inspection, these regions of similarity were also found on the Cdc34 protein. The similarity of Ubs1 to these Ub-conjugating enzymes is confined to two short noncontiguous regions we have called A and B (Fig. 7). With regard to Ubs1, region A exhibits 65% similarity with Cdc34 and 79% with Ubc1. In region B, Ubs1 shows 74% similarity with Cdc34 and 76% with Ubc1. Percent similarity was computed by assigning a numerical similarity value to each pair of amino acids aligned within regions A and B (3). A total of these values was expressed as the percentage of a hypothetical score obtained by perfectly matched residues. On the basis of these analyses, we conclude that the region of similarity shared between Ubs1 and either Ubc1 or Cdc34 is significant.

From the similarity analysis and using the previously determined crystallographic structure of the *Arabidopsis* Ubc1 protein (7) as a model of Cdc34, we have mapped these two regions onto its three-dimensional structure (Fig. 8). Although these regions are separated from one another in the Cdc34 primary sequence by 14 residues and in the Ubs1 primary sequence by 66 residues, they fold in three dimensions to produce a defined patch on the surface of the conjugating enzyme. Interestingly, the *cdc34-2* substitution which is nor-

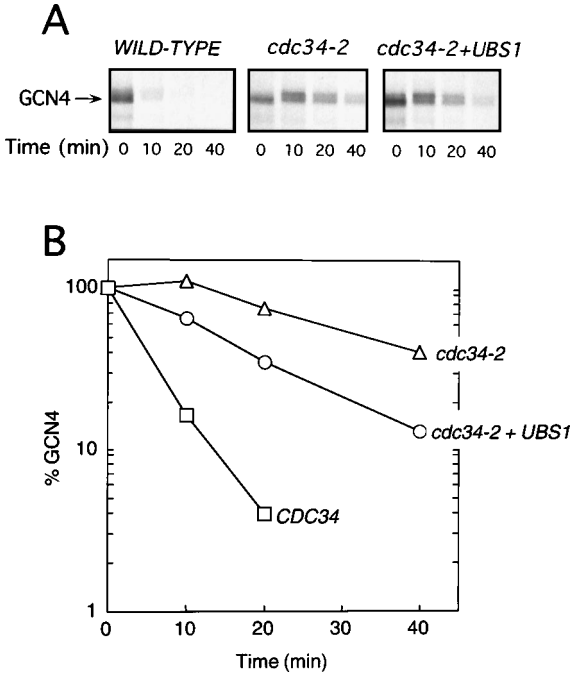

FIG. 6. Partial stabilization of Gcn4 by *UBS1* overexpression. (A) A sodium dodecyl sulfate gel autoradiogram of radiolabelled Gcn4–β-galactosidase derived from either wild-type cells, *cdc34-2* mutant cells, or *cdc34-2* mutant cells carrying the *UBS1* plasmid that was immunoprecipitated from cell extracts at the indicated times following the radioactive pulse. The Gcn4–β-galactosidase fusion protein (Gcn4) migrates as several distinct electrophoretic species (27). (B) A quantitation of the combined radioactivity of the Gcn4–β-galactosidase bands shown in panel A plotted against time. Values are expressed as a percentage of the time zero value.

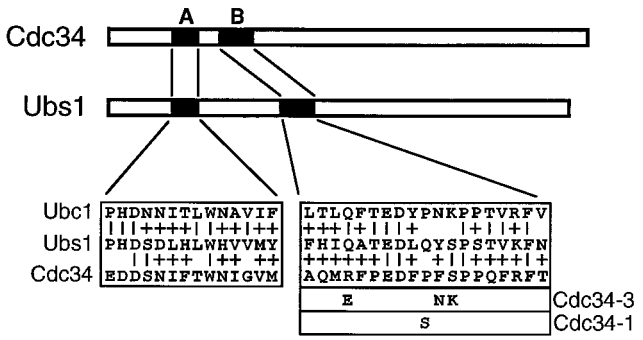

FIG. 7. Two regions of Ubs1 exhibit strong similarity to the Ub-conjugating enzymes Cdc34 and wheat Ubc1. The upper portion of this figure represents the linear peptide sequence of the Ubs1 and Cdc34 proteins, showing the relative sizes and positions of the similar regions (black boxes). The lower portion of this figure displays an alignment of these regions for Cdc34, wheat Ubc1, and Ubs1. The region closest to the amino terminus represents residues 34 to 47 of Cdc34 (18), 28 to 41 of Ubc1 (43), and 31 to 44 of Ubs1. The second region represents residues 62 to 80 of Cdc34, 55 to 73 of Ubc1, and 111 to 129 of Ubs1. A perfect residue match between sequences is indicated by a vertical line, whereas nonidentical conservation between sequences is indicated by a plus sign. Amino acid substitutions caused by the *cdc34-1* and *cdc34-3* mutations are indicated below the wild-type Cdc34 sequence.

mally suppressed by *UBS1* lies outside of the Ubs1-related patch. We reasoned therefore that if this region functioned in *UBS1* suppression, then amino acid substitutions made within this region should be detrimental to Cdc34 in a manner that could not be suppressed by *UBS1*. To test this idea, we examined the behavior of the *cdc34-1* allele carrying a single substitution situated within the patch (Pro-71 to Ser) and a newly created *cdc34* derivative (*cdc34-3*) carrying three substitutions within the patch (Arg-65 to Glu, Phe-72 to Asn, and Ser-73 to Lys). To minimize possible perturbations to the structure of Cdc34, substitutions were confined to positions situated on the surface and correspond to residues found in another yeast E2 of different function, Ubc2. Upon examining *cdc34* mutant cells at their allele-specific nonpermissive temperatures (Materials and Methods), *UBS1* overexpression was found to reduce the frequency of multibudded cells by 74, 7, and 0% for the *cdc34-2*, *cdc34-1*, and *cdc34-3* mutant alleles, respectively. Therefore, *UBS1* could suppress the *cdc34-2* mutation readily, the *cdc34-1* allele only poorly, and the *cdc34-3* allele not at all. Thus, *UBS1* shows allele-specific suppression of ts *cdc34* alleles and specifically an impaired ability to suppress *cdc34* mutations that reside within the region of similarity that Cdc34 shares with Ubs1. Significantly, the phenotypic effects of the *cdc34-3* allele are less severe than those of *cdc34-1* or *cdc34* alleles even though it bears the greatest mutational load. Therefore, the allele specificity exhibited by *UBS1* expression does not correlate with the strength of the mutant phenotype. On the basis of this observation, we conclude that the allele-specific behavior of *UBS1* expression reflects a mechanistic link between Cdc34 and Ubs1 in which the region of similarity between these proteins plays a key role.

DISCUSSION

In the present work, we describe a new yeast gene, *UBS1*, whose function is mechanistically linked to the function of Cdc34. The evidence that establishes the functional relationship between Ubs1 and Cdc34 is based on two observations. *UBS1* overexpression suppresses both the cell cycle and Gcn4 turnover defects of a *cdc34* ts mutant. Conversely, the deletion of *UBS1* greatly accentuates the defects associated with this mutant. *UBS1* expression therefore has a positive effect on processes that are directly related to Cdc34 function.

The mechanistic link between the Ubs1 and Cdc34 proteins is based on several pieces of evidence which demonstrate that *UBS1* expression posttranslationally modifies the activity of the Cdc34 polypeptide. First, the fact that *UBS1* expression directly affects the turnover of Gcn4 (Fig. 6) illustrates that Ubs1 positively influences Cdc34-dependent proteolysis, thereby discounting less direct mechanisms of suppression including the possibility that *UBS1* expression lowers the levels of Cdc34 targets by nonproteolytic means or alternatively that *UBS1* expression somehow bypasses the need to target these substrates altogether. Second, the observation that Ubs1 expression has no effect on the steady-state levels of Cdc34-2 (Fig. 5) at the nonpermissive temperature indicates that *UBS1* expression contributes to the activity of the Cdc34 polypeptide, without altering its intracellular levels. Finally, although Ubs1 and Cdc34 exhibit no overall similarity to one another, they do hold structural attributes in common. Ubs1 exhibits significant similarity to a defined patch on the surface of Cdc34. Whereas *cdc34* mutations that are unrelated to the patch are suppressed by *UBS1* expression, mutations that affect the patch lose their dependence on Ubs1. Thus, the functional relationship between Cdc34 and Ubs1 correlates well with their limited structural similarity.

It is unlikely that Ubs1 functions redundantly as an E2 that functionally overlaps with Cdc34, on the basis of two observations. With the exception of the limited similarity between Ubs1 and Cdc34 already discussed, Ubs1 shares no significant similarity with the conserved catalytic domains of any E2 identified to date. Furthermore, if Ubs1 exhibited some of the catalytic properties of Cdc34, it would be expected to exert its suppressive effects on all three of the *cdc34* mutant alleles examined here. This notion is clearly at odds with the allele-specific behavior observed for Ubs1.

Suppressive effects that are allele specific have often been cited as genetic evidence for the physical interaction of the suppressor and mutant protein (5, 29). Although we cannot rule out an interaction between Ubs1 and Cdc34, we have been

FIG. 8. Three-dimensional location of the region of similarity shared between the Cdc34 and Ubs1 proteins. Shown is a CPK and ribbon image of *Arabidopsis* Ubc1-conjugating enzyme (7) used here as a model of the Cdc34 catalytic domain. Indicated in pink is the region of similarity between Cdc34 and Ubs1 as described for Fig. 7; the catalytic cysteine residue is indicated in yellow. The position of the *cdc34-1* substitution (Pro-71 to Ser) is indicated in red.

unable to detect their interaction either in vitro or with the two-hybrid system (results not shown). There is, however, an equally plausible alternative that is consistent with the data presented here and can account for the suppressive behavior of *UBS1*. Cdc34 and Ubs1 may independently interact with an inactivator of Cdc34 via the patch they hold in common. Increased expression of *UBS1* would be expected to sequester the inactivator, thereby elevating the levels of active Cdc34. Experiments to test this hypothesis are under way.

Recently, we have found that the region corresponding to the Ubs1-Cdc34 patch in other E2s plays a role in determining their functional distinctiveness. This evidence is based on our observation that transposition of amino acid positions within the B region of the Rad6 protein with their Ubc4 counterparts confers partial Ubc4 function on Rad6 (unpublished data). The temperature sensitivity of the Cdc34-3 polypeptide that results from surface substitutions in the same region is consistent with this view. The possibility that a structural determinant of E2 specificity may also contribute to its regulation would provide a logical and attractive means for differential E2 regulation.

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