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A Ubiquitin-Conjugating Enzyme in Fission Yeast That Is Essential for the Onset of Anaphase in Mitosis

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A cDNA encoding a ubiquitin-conjugating enzyme designated UbcP4 in fission yeast was isolated. Disruption of its genomic gene revealed that it was essential for cell viability. In vivo depletion of the UbcP4 protein demonstrated that it was necessary for cell cycle progression at two phases, G2/M and metaphase/anaphase transitions. The G2 arrest of UbcP4-depleted cells was dependent upon *chk1*, which mediates checkpoint pathway. UbcP4-depleted cells arrested at metaphase had condensed chromosomes but were defective in separation. However, septum formation and cytokinesis were not restrained during the metaphase arrest. Overexpression of UbcP4 specifically rescued the growth defect of *cut9*^{ts} cells at a restrictive temperature. *cut9* encodes a component of the anaphase-promoting complex (APC) which is required for chromosome segregation at anaphase and moreover is defined as cyclin-specific ubiquitin ligase. Cdc13, a mitotic cyclin in fission yeast, was accumulated in the UbcP4-depleted cells. These results strongly suggested that UbcP4 is a ubiquitin-conjugating enzyme working in conjunction with APC and mediates the ubiquitin pathway for degradation of "sister chromatid holding protein(s)" at the onset of anaphase and possibly of mitotic cyclin at the exit of mitosis.

Rapid protein degradation catalyzed by the ubiquitin-dependent 26S proteasome pathway has been shown to be involved in cellular regulation such as cell cycle control, DNA repair, stress response, and transcriptional control (for recent reviews, see references 6 and 24). The proteasome degrades proteins containing covalently attached multiubiquitin chains. Ubiquitination of proteins is a multiple enzymatic process. Ubiquitin, activated by the ubiquitin-activating enzyme (E1), is attached to the same enzyme by a high-energy bond and then transferred to the ubiquitin-conjugating enzyme (E2 or Ubc). While in many cases ubiquitin can be directly transferred from E2 and covalently attached to a substrate protein, ubiquitination of some proteins requires the additional activity of ubiquitin ligase (E3). Most cells contain a single E1 enzyme, but E2 exists as a family of enzymes. In budding yeast, at least 12 genes for E2 have been identified, each of which is involved in different cellular functions (24, 28). Different classes of E3 have been identified, such as Ubr1 (4), hectodomain proteins (26, 38, 40), and the anaphase-promoting complex (APC [see below]) (27, 32, 43, 44). Thus, E2, alone in some cases but often along with its partner E3, recognizes and catalyzes the conjugation of ubiquitin to a specific or preferred substrate.

Mitosis is initiated in most organisms by synthesis and the accumulation of mitotic cyclin which regulates cdc2 kinase, a catalytic subunit of the mitosis-promoting factor. For the exit from mitosis, degradation of mitotic cyclin is necessary and is shown to be mediated by the ubiquitin-proteasome pathway (11, 14, 15, 21, 34, 37). Ubiquitination of cyclin requires a specific signal sequence, called the destruction box, in the cyclin itself. The components used for cyclin ubiquitination have been characterized. Cyclin-specific E3 has been identified biochemically as a large complex, termed cyclosome in clam egg extract (43) or APC in mammalian cultured cells (44), *Xenopus* egg extract (32), and yeast (27). APC is composed of multiple components including the homolog of budding yeast Cdc16, Cdc23, and Cdc27. Yeast mutants with mutations in the genes for these APC components cause cell cycle arrest in mitosis with a short spindle and unseparated chromosome. Thus, APC is required at the onset of anaphase for sister chromatid separation, which is accomplished not by cyclin destruction but by degradation of a protein(s) essential for sister chromatid pairing during metaphase (25). Recently, it was shown that APC-dependent proteolysis of the Cut2 protein is required for sister chromatid separation in fission yeast (13). E2, which is responsible for ubiquitination of cyclin B, has been enzymatically purified and identified as UBC-x in *Xenopus* egg extract (48) and E2-c in clam egg extract (3). Analysis of the cDNAs of these E2s revealed that they are homologous to each other but not to any of the E2s identified so far in budding yeast and other species. They ubiquitinate cyclin B in an APC-dependent fashion in vitro. However, it is not clear whether the two E2s are involved in chromosome segregation at the onset of anaphase. Among newly isolated genes in this study of E2 in *Schizosaccharomyces pombe*, *ubcP4*$^{+}$ was found to be highly homologous to the cyclin-specific E2s, UBC-x and E2-c, and proved to be essential for the transition from metaphase to anaphase in mitosis. Cdc13, a mitotic cyclin of the fission yeast, was stabilized in UbcP4-depleted cells. Overproduction of UbcP4 in the cell specifically suppressed the temperature-sensitive mutation defect in the component of APC, suggesting that UbcP4 mediates the ubiquitin pathway specific to APC.

MATERIALS AND METHODS

Strains and media. The *S. pombe* strains used in this study are listed in Table 1. Cells were grown in complete medium (YE medium) or minimal medium (MM) supplemented with amino acids, uracil, or adenine as described in reference 31.

Screening cDNAs. The cDNA expression library was constructed with wild-type *S. pombe* 972 mRNA and a ZAP-cDNA Synthesis Kit (Stratagene) as specified by the manufacturer. Double-stranded cDNAs were cloned into the *Eco*R-I-*Xho*I site of Bluescript II SK(−) and transformed to *Escherichia coli* XL1-Blue MRF′. For the screening of ubiquitin binding, 100 μl of cell culture from 50 independent colonies were mixed, washed, and suspended in 50 μl of 50 mM Tris-HCl (pH 7.4)–1 mM dithiothreitol (DTT), for lysis by sonication. The

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

Strain	Genotype
FO100	*h*$^+$/*h*$^-$ *his2*$^-$/*his2*$^+$ *leu1-32/leu1-32 ura4D18/ura4D18 ade6-M216/ade6-M210*
FO101	Same as FO100 except *ubcP4*::*ura4*$^+$/*ubcP4*$^+$
FO102	Same as FO100 except (*chk1-ubcP4*)::*ura4*$^+$/(*chk1-ubcP4*)$^+$
FO201	*h*$^-$ *leu1-32 ura4D18 ade6-M210 ubcP4::ura4*$^+$ [*pRep81-ubcP4*$^+$ *LEU2*]
FO202	*h*$^-$ *leu1-32 ura4D18 ade6-M210 ubcP4*::*ura4*$^+$ [*pSP1-ubcP4*$^+$ *LEU2*]
FO301	*h*$^-$ *leu1-32 ura4D18 ade6-M210* (*chk1-ubcP4*)::*ura4*$^+$/(*chk1-ubcP4*)$^+$ [*pRep81-ubcP4*$^+$ *LEU2*]
FO302	Same as FO301 except [*pSP1-chk1*$^+$ *ade6*$^+$]
cut9^{ts}	*h*$^-$ *leu1-32 cut9-665*
cdc25^{ts}	*h*$^-$ *leu1-32 cdc25-22*

lysate was microcentrifuged for 20 min at 15,000 rpm (18,500 × *g*) at 4°C. A 10-μl volume of the supernatant was used for the ubiquitin-binding assay in 30 μl of reaction mixture, which consisted of 50 mM Tris-HCl (pH 7.4), 1 mM ATP, 10 mM $MgCl_2$, 0.5 mM DTT, 0.15 U inorganic pyrophosphatase, 50 ng of purified *S. pombe* E1, and ^{32}P-labeled ubiquitin (10^5 cpm). After incubation at 30°C for 5 min, the reaction was terminated by adding 30 μl of 4% sodium dodecyl sulfate SDS, 4 M urea, 0.2% bromophenol blue (BPB) and analyzed by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide). The ^{32}P-ubiquitin–E2 complex was detected by autoradiography.

The E1 enzyme was purified from the *S. pombe* cell extract by a combination of DEAE chromatography, ubiquitin affinity chromatography, and gel filtration as previously described (7, 22). Ubiquitin cDNA was fused to the protein kinase A (PKA)–glutathione *S*-transferase site in the pGEX-2TK vector (Pharmacia), and the fusion protein was bacterially expressed, purified with glutathione-Sepharose 4B (Pharmacia), and labeled with the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP (30). The labeled ubiquitin was purified by centrifugation after cleavage with thrombin.

Plasmid construction and genetic manipulations. The cDNA for *Arabidopsis thaliana* ubiquitin was a gift from J. Caris, University of California, Davis. *Bam*HI and *Eco*RI sites before and after the coding sequence, respectively, were introduced by PCR, and the *Bam*HI-*Eco*RI fragment was fused in frame to Glutathione *S*-transferase and the PKA site in the pGEX-2TK vector (Pharmacia).

For bacterial expression of UbcP1, UbcP2, UbcP3, and UbcP4, the *Bam*HI and *Eco*RI sites were introduced by PCR before and after the coding sequence of the each Ubc, respectively, and then ligated in frame to the pGEX-2TK vector.

Genomic *ubcP4* was initially cloned in Bluescript II SK(−) as an *Xba*I-*Xho*I fragment of 3.5 kb. For disruption, the *Hin*cII-*Cla*I fragment was replaced with a 2-kb *Hin*dIII fragment containing the *ura4*$^+$ gene, removing the amino acid sequence from Asp_{25} through the C terminus of UbcP4. DNA linearized by restriction with *Xho*I transformed FO100 cells to disrupt the *ubcP4* gene.

The complete coding sequence for *chk1* was cloned in Bluescript II SK(−) by PCR and then recombined in vitro at the *Xba*I site with the *Xba*I-*Xho*I genomic fragment containing *ubcP4*. For disruption, the *Xba*I-*Cla*I fragment was replaced with the *ura4*$^+$-containing fragment, which removed the sequence from the N terminus through Arg_{321} in Chk1 and the whole sequence of UbcP4. The DNA was linearized by *Xho*I restriction, and then it transformed FO100 cells to disrupt both the *chk1* and *ubcP4* genes.

For the cloning of cDNA for UbcP4 in the pREP1, pREP41, and pREP81 expression vectors (5, 35) and of cDNA for UbcP1 and UbcP2 in pREP41, the ATG and stop codons of each gene were fused to the *Nde*I and *Sal*I sites, respectively, by PCR. Afterwards, the *Nde*I-*Sal*I fragments replaced the *Nde*I-*Sal*I fragment in the pREP vectors.

Culture conditions. For in vivo depletion of UbcP4, FO201 cells were grown at 32°C in thiamine-free minimal medium (1) containing adenine (75 μg/ml) to a cell density of 5×10^6 cells/ml, and then thiamine was added at 10 mM for all experiments. Continuous cell growth was maintained through dilution, keeping the cell density between 10^5 and 10^7 cells/ml, and the number of cells was determined with a hemocytometer. For Western blotting, 5×10^7 cells were harvested, and the extracts were prepared with glass beads as described by Kaiser et al. (31).

To synchronize *cdc25*^{ts} cells, cells grown to 2×10^6 cells/ml at 25°C were transferred to 35°C for 4 h in YE medium and then released by shifting down to 25°C. Synchronization was monitored by measuring the septation index.

The *cut9*^{ts} strain (39), which was a gift from M. Yanagida, Kyoto University, was transformed with the cDNAs for Ubc in pREP-vectors. Transformation was carried out by the lithium acetate method, and transformed cells were selected at 25°C on an MM agar plate containing 10 mM thiamine (1). For the suppression test, cells harboring the plasmid were inoculated on MM agar with or without 10 mM thiamine and incubated at 25 or 34°C for 6 days.

Antibodies and immunofluorescence. The anti-UbcP4 antibody was raised against bacterially expressed protein. The protein was first purified as a fused version of glutathione *S*-transferase–UbcP4 by using glutathione-Sepharose beads and was then further separated by SDS-polyacrylamide gel electrophoresis after digestion with thrombin. A 20-μg portion of the protein excised from the gel was emulsified with incomplete Freund's adjuvant and injected into rats every 2 weeks for 2 months.

The antibody against Cdc13 was prepared by immunizing rabbits with bacterially produced Cdc13 protein. His-tagged Cdc13 protein was purified from *E. coli* extract on an Ni^{2+} affinity column. The protein was further purified by SDS-gel electrophoresis and emulsified with incomplete Freund's adjuvant for injection every 2 weeks for 2 months.

Anti-PSTAIRE antibodies were purchased from Santa Cruz Biotechnology, Inc.

Cells fixed with 2.5% glutaraldehyde were stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Indirect immunofluorescence of tubulin was performed with the monoclonal antibody TAT1-1 (19) (kindly given by O. Niwa, Kazusa DNA Research Institute) as previously described (47). Cells of each phenotype were counted in random pictures of stained cells obtained at the indicated times after thiamine addition.

Histone H1 kinase assay. Histone H1 kinase activity was assayed basically by the method of Moreno et al. (36). A total of 2×10^8 cells were collected by filtration and washed with ice-cold stop solution (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF). The cells were finally suspended in 100 μl of ice-cold HB buffer, which is composed of 25 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium orthovanadate (Sigma), 15 mM nitrophenylphosphate (Sigma), 1 mM phenylmethylsulfonyl fluoride, 60 mM β-glycerophosphate (Sigma), 20 μg of leupeptin (Sigma) per ml, and 40 μg of aprotinin (Sigma) per ml. Glass beads (Sigma; 425 to 600 μm in diameter) were added, and the mixture was vigorously vortexed for 30 s six times to disrupt cells. A 1-ml volume of HB buffer was added, and the S30 extract, usually with a protein content of about 300 μg/ml, was obtained by ultracentrifugation. Phosphorylation was carried out at 30°C for 20 min in 20 μl of HB buffer containing 4 μl of the extract (about 1.2 μg of proteins), 5 mg of histone H1 (Boehringer Mannheim) per ml, 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol [DuPont-NEN]), 100 μM ATP, and 2.5 mM EGTA. The reaction was stopped by adding the loading buffer and propagated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) followed by autoradiography and quantification with a Fujix Bas2000 image analyzer.

Flow cytometry. Cells (2×10^7) were fixed with 70% (vol/vol) ethanol, treated with DNase-free RNase A (100 μg/ml), and stained with 0.05% propidium iodide. Samples of 20,000 cells were analyzed with a FACScan (Becton Dickinson).

Nucleotide sequence accession numbers. The DNA sequences described in this report have been deposited in the EMBL/GenBank/DDBJ database under accession no. D85544 and D85545.

RESULTS

Isolation of cDNAs and genes for Ubc in *S. pombe*. The cDNAs for a family of E2s in *S. pombe* were isolated by screening the cDNA gene products expressed in *E. coli* cells according to their ability to bind activated ubiquitin in vitro. Of 20,000 independent cDNA clones screened, 20 produced a protein which forms a thiolestel bond with ubiquitin in the presence of the ubiquitin-activating enzyme (Fig. 1a). Subsequent sequencing analysis identified four types of E2 cDNA. The predominant two isolates (nine each) encoded UbcP1 and UbcP2, while two rare isolates encoded UbcP3 and UbcP4. Figure 1b indicates that bacterially expressed UbcP1, UbcP2, UbcP3, and UbcP4 proteins were able to accept ubiquitin in vitro in an E1- and ATP-dependent fashion. In the absence of ATP, the radioactivities associated with the Ubc proteins were much reduced but not thoroughly diminished compared with that of the complete mixture. We supposed that this was due to a trace of ATP carried over from the E1 and/or E2 preparation, obtained from *S. pombe* and *E. coli* extract, respectively. Two forms of UbcP3 were detected, which we did not further characterize.

The four Ubcs showed 30 to 40% identities with each other, and they all contained a UBC domain with a catalytic cystein residue (Fig. 2), which are common to all known E2s. UbcP1 and UbcP2 were found to be homologs of Ubc4/5 (9) and

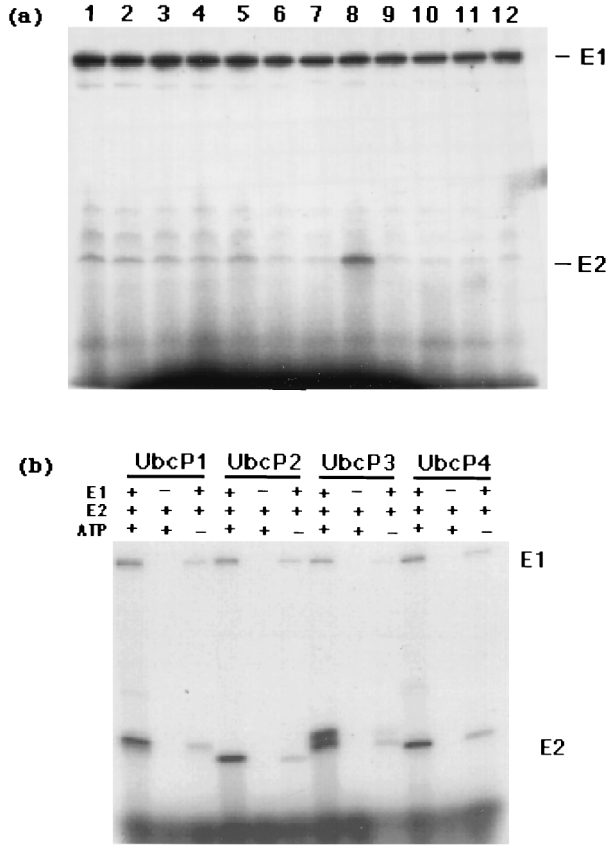

FIG. 1. Enzymatic activity of Ubc proteins. (a) Example of screening gel electrophoresis as described in Materials and Methods. Each lane contained *E. coli* extracts from 50 transformants with the *S. pombe* cDNA library. In this case, among 600 clones totally screened, a Ubc candidate was found in lane 8. Faint background was observed because of a trace of E2 enzymes contaminating the activating enzyme preparation. E1 and E2 indicate the positions of the ubiquitin-activating enzyme and Ubc, respectively, associated with ^{32}P-labeled ubiquitin. (b) Bacterially expressed UbcP1, UbcP2, UbcP3, and UbcP4 proteins were examined with their acceptor activity of ^{32}P-labeled ubiquitin in the presence (+) or absence (−) of ubiquitin-activating enzyme and ATP, as described in Materials and Methods. E1 and E2 are indicated as in panel a.

Ubc2/Rad6 (29), respectively, in *Saccharomyces cerevisiae*, sharing more than 60% identity. UbcP3 was highly homologous to Ubc7 in *S. cerevisiae* (28), while UbcP4 was novel. In addition to the UBC domain, UbcP4 contained an extension sequence of 30 amino acids at its N terminus (Fig. 2), which was a distinctive feature. Hence, hereafter we concentrate on the function of UbcP4.

The genomic clone for *ubcP4*$^+$ was isolated from the genomic library by using the cDNA as a probe. Sequence analysis and comparison with that of the cDNA identified four introns in the *ubcP4*$^+$ coding sequence (Fig. 3). A search of the database revealed that the sequence upstream of the *ubcP4* genomic sequence was complementary to the *chk1* gene (46), so that the two genes were encoded about 250 bp apart from each other in the opposite directions (Fig. 3).

***ubcP4* is essential for cell growth.** In diploid FO101 cells (Table 1), a *ubcP4*$^+$ gene was disrupted by replacing a majority of its coding sequence with the *ura4*$^+$ gene (Fig. 3), which was confirmed by both genomic Southern blotting and PCR (data not shown). A conventional tetrad analysis of this diploid strain resulted in only two viable ura$^-$ and no ura$^+$ colonies from each ascus, indicating that haploid cells with the disrupted *ubcP4* gene were nonviable (data not shown). Thus, *ubcP4* is essential for growth.

A haploid strain, FO201 (Table 1), was constructed, in which the cDNA for UbcP4 was conditionally expressed under the control of a thiamine-repressible promoter in pREP81 (5, 35), while an endogenous *ubcP4*$^+$ gene was disrupted. FO201 cells grew normally in thiamine-free medium, producing five times more UbcP4 protein than in wild-type cells (Fig. 4B, compare lanes 1 and 2), with a similar doubling time and cellular morphology to those in the wild type. Western blot analysis with a synchronized cell culture indicated that the amount of UbcP4 protein in wild-type cells did not fluctuate during the cell cycle (data not shown). These results allowed us to use the artificially constitutive expression of UbcP4 under the control of the pREP vector system.

After the addition of thiamine to the medium, FO201 cells continued to grow for the first 14 h and then stopped dividing (Fig. 4A). Western blot analysis with an antibody against UbcP4 showed that the intracellular level of the UbcP4 protein after thiamine repression decreased and was not detectable at the time of growth arrest (Fig. 4B). After thiamine repression of the nmt1 promoter, several generations elapsed before the growth arrest occurred. It might be necessary for artificially overproduced UbcP4 protein to be diluted out. This period did not seem to be exceedingly long for nmt1-repression experiments.

Cell cycle arrest of UbcP4-depleted cells. To clarify whether the growth arrest from UbcP4 depletion occurred at specific cell cycle stages, the phenotypes of the thiamine-repressed FO201 cells were examined by DAPI staining. Up to 14 h after the addition of thiamine, no abnormal cells appeared, which was in good agreement with the growth of the UbcP4-depleted cells as described above. After 14 h, simultaneously with growth arrest (Fig. 4A), cells interfering with M-phase progression appeared (Fig. 5). Abnormalities were classified into the following types. Type A cells showed metaphase arrest with condensed chromosomes. In type B cells, about 80% of which had condensed chromosomes, the septum was formed but chromosome separation did not occur. Type C cells showed a mitotic catastrophe known as the *cut* phenotype (23, 45). All types had a short metaphase spindle associated with condensed chromosomes (Fig. 5, lower panels). The population of cells for each phenotype is summarized in Table 2. Displacement of the mitotic nuclei was another distinctive feature in these cells. Consequently, about 10% of the total cells were anucleated, and binucleated cells were also found. Flow cytometry analysis showed that the majority of cells had a 2C DNA content at 14 h after thiamine addition (see Fig. 7). These results indicated that UbcP4-depleted cells were defective for the onset of anaphase in terms of sister chromatid separation and also for the restraint of septum formation and cytokinesis during metaphase and anaphase. After prolonged (16-h) thiamine repression of cDNA for UbcP4 in FO201 cells, elongated cells began to appear (Fig. 5 to 7). The size distribution of FO201 cells grown under the presence of thiamine for 18 h was analyzed and is shown in Fig. 6. Compared with cells expressing UbcP4, the longer cells obviously accumulated in the UbcP4-depleted culture. On the basis of nuclear and microtubule structures, two types of elongated cells were elucidated: metaphase cells (type D) with condensed chromosomes and short metaphase spindles, and interphase cells (type E). The size distribution and DNA content of the arrested cells were analyzed by flow cytometry, indicating that the majority of elongated cells had a 2C DNA content (Fig. 7). Scoring under the microscope showed that about half of the elongated cells after an 18-h depletion of UbcP4 were arrested during metaphase (type D)

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                    10         20         30         40         50
Ubc2/Rad6     1 MSTPAR---- ---------- ---------- RRLMRDFKRM KEDAPPGVSA
UbcP1         1 MA-------- ---------- ---------L KRINRELADL GKDPPSSCSA
UBCP2         1 MSTTAR---- ---------- ---------- RRLMRDFKRM QQDPPAGVSA
UbcP3         1 MSKAMP---- ---------- ---------L RRLMKEYKEL TENGPDGITA
UbcP4         1 MDSDMQNQNP HTNSKNSSSA GMAVDGHSVT KRLRSELMSL MMSNTPGISA
Hus5          1 MSSLCKT--- ---------- ---------- -RLQEERKQW RRDHPFGFYA

                   60         70         80         90        100
Ubc2/Rad6    51 SPLPDNV--- -MV-WNAMII GPADTPYEDG TFRLLLEFDE EYPNKPPHVK
UbcP1        51 GPVGD-D--- -LFHWQATIM GPADSPYAGG VFFLSIHFPT DYPFKPPKVN
UBCP2        51 SPVSDNV--- -ML-WNAVII GPADTPFEDG TFKLVLSFDE QYPNKPPLVK
UbcP3        51 GPSNED---- DFFTWDCLIQ GPDGTPFEGG LYPATLKFPS DYPLGPPTLK
UbcP4        51 FPDSDSN--- -LLHWAGTIT GPSDTYYEGL KFKISMSFPA NYPYSPPTII
Hus5         51 KPCKSSDGGL DLMNWKVGIP GKPKTSWEGG LYKLTMAFPE EYPTRPPKCR

                  110        120        130        140        150
Ubc2/Rad6   101 FLSEMFHPNV YANGEICLDI LQ-------- ---NR--WTP TYDVASILTS
UbcP1       101 FTTRIYHPNI NSNGSICLDI LR-------- ---DQ--WSP ALTISKVLLS
UBCP2       101 FVSTMFHPNV YANGELCLDI LQ-------- ---NR--WSP TYDVAAILTS
UbcP3       101 FECEFFHPNV YKDGTVCISI LHAPGDDPNM YESSSERWSP VQSVEKILLS
UbcP4       101 FTSPMWHPNV DMSGNICLDI LK-------- ---DK--WSA VYNVQTILLS
Hus5        101 FTPPLFHPNV YPSGTVCLSI LN-------- ---EEEGWKP AITIKQILLG
                             *
                  160        170        180        190        200
Ubc2/Rad6   151 IQSLFNDPNP ASPANVEAAT LFKDHKSQYV KRVKETVEKS WEDDMDDMDD
UbcP1       151 ICSLLTDPNP DDPLVPEIAH VYKTDRSRYE LSARE----- WTRKYA-I--
UBCP2       151 IQSLLNDPNN ASPANAEAAQ LHRENKKEYV RRVRKTVEDS WES-------
UbcP3       151 VMSMLAEPND ESGANIDACK MWREDREEYC RVVRRLARKT L---------
UbcP4       151 LQSLLGEPNN ASPLNAQAAE LWSKDPIEYK RLLMQ----- RYKEIDEI--
Hus5        151 IQDLLDDPNI ASPAQTEAYT MFKKDKVEYE KRVRA----- ----------

                  210
Ubc2/Rad6   201 DDDDDDDDDD DEAD
UbcP1       201 ---------- ----
UBCP2       201 ---------- ----
UbcP3       201 ---------- --GL
UbcP4       201 ---------- ----
Hus5        201 -------QAR ENAP
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FIG. 2. Comparison of Ubcs. Sequences deduced from *S. pombe* cDNAs for UbcP1, UbcP2, UbcP3, UbcP4, and Hus5 (2), together with that of *S. cerevisiae* Ubc2/Rad6 (29), were aligned. Identical amino acids are shaded, and a dash indicates the absence of a corresponding residue. A cysteine residue in the UBC domain for thiolester formation with ubiquitin is marked by an asterisk.

and the other half were arrested during interphase (type E). Few cells, however, which elongated and contained less than a 2C content of DNA were detected by the flow cytometry analysis (Fig. 7). These indicate that the type E cells had a 2C DNA content and were arrested at the G_2 phase.

In plasmid loss experiments with FO202 cells, in which the chromosomal *ubcP4*$^+$ was disrupted and the *ubcP4*$^+$ gene was on plasmid vector pSP1 (8), all types of arrested cells were observed after growth under nonselective conditions for pSP1 (data not shown). Spores of a diploid strain (FO101) carrying *ura4/ura4* and heterozygous *ubcP4*::*ura4*$^+$/*ubcP4*$^+$ genes were germinated in the absence of uracil, resulting in type A, B, D, and E cells (data not shown). Thus, we concluded from the phenotype analysis that UbcP4 was required for two steps during the cell cycle, transition from the G_2 phase to the M phase and transition from metaphase to anaphase.

Histone H1 kinase activity increases in UbcP4-depleted cells. It had been reported that overexpression of dominant negative mutations of *cdc2* can cause both G_2 arrest and interruption of chromosome segregation, in which case cdc2 kinase activity is suppressed (12, 33). To verify this point, histone H1 kinase activity in UbcP4-depleted cells was examined. As shown in Fig. 8A, thiamine repression of UbcP4 cDNA in the FO201 cells for 16 h resulted in an increase in histone H1 kinase activity. This was in parallel with the accumulation of metaphase-arrested cells although was not uniformly synchronized. Quantification of the incorporated radioactivity indicated that kinase activity in the UbcP4-depleted cells was four to five times higher than that in asynchronously growing cells. The level of histone H1 kinase activity in FO201 cells after 16 or 18 h of thiamine repression was compared with

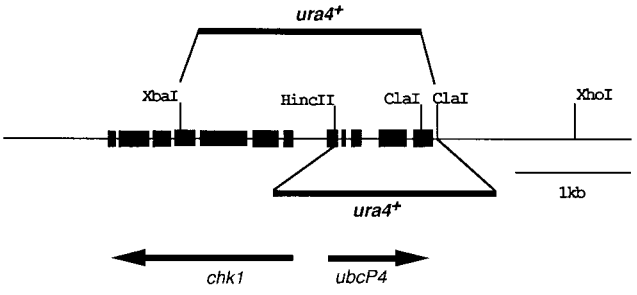

FIG. 3. Structure and disruption of the *chk1-ubcP4*$^+$ region. The coding sequences of the *ubcP4* and *chk1* genes, consisting of five and seven exons, respectively, are shown by solid boxes together with some unique restriction sites. Arrows indicate the transcription of each gene. For disruption of *ubcP4*, the *Hin*cII-*Cla*I fragment was replaced with the *ura4*$^+$ fragment (lower line). Disruption of both *chk1* and *ubcP4* was accomplished by replacing the fragment between the *Xba*I site in *chk1* and the *Cla*I site after *ubcP4* with the *ura4*$^+$ fragment (upper line).

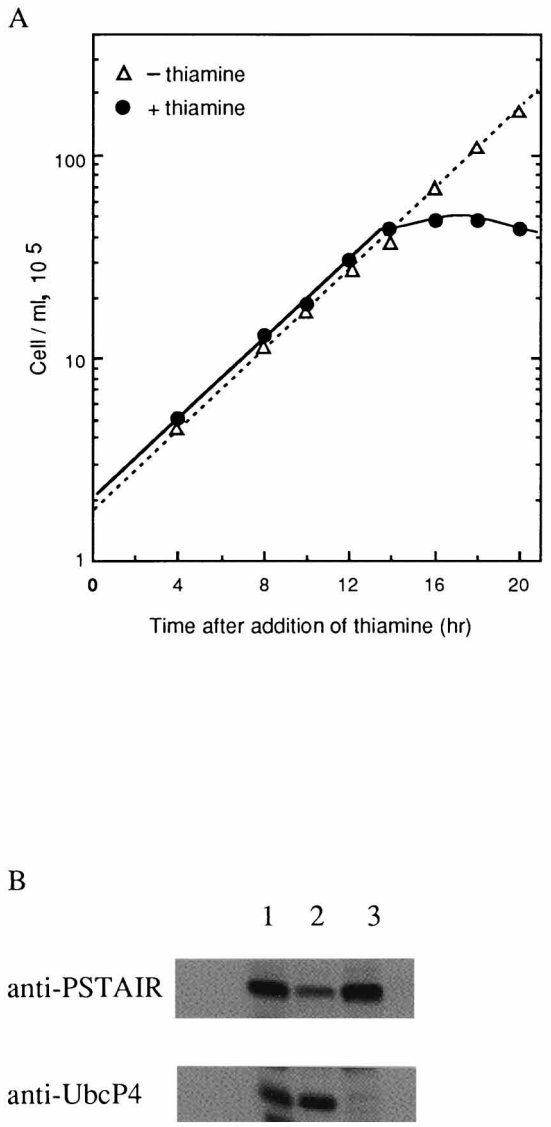

FIG. 4. (A) Growth arrest of UbcP4-depleted cells. Cells of the FO201 strain were grown in MM in the presence (solid line) or absence (broken line) of thiamine. Thiamine was added at time zero. (B) Loss of the UbcP4 protein. A Western blot of crude lysates of cells, probed with the same filter with anti-UbcP4 (bottom panel) or anti-PSTAIRE antibody (top panel) as a quantitative control. Lanes: 1, wild-type haploid strain; 2, FO201 cells growing in thiamine-free medium; 3, FO201 cells grown for 14 h with thiamine.

that in cells synchronized at the M phase. *cdc25*^{ts} cells were arrested at the G_2 phase by a temperature shift to 35°C and then released to the M phase. We found that 60% of *cdc25*^{ts} cells were synchronized at the M phase (Fig. 8C) and, at 20 min after the temperature release, fully expressed cdc2 kinase activity (Fig. 8B). The histone H1 kinase activity in the UbcP4-depleted cells was about half of that in *cdc25*^{ts} cells synchronized at the M phase and expressing maximum cdc2 kinase (Fig. 8B and C). These indicated that cdc2 kinase activity was not confined to UbcP4-depleted cells.

Suppression of APC mutants. The interruption of the metaphase/anaphase transition in UbcP4-depleted cells was strikingly similar to the phenotype of a series of *cut* mutants. One of the *cut* genes, *cut9* (39) is a counterpart of *CDC16*$^{+}$ in *S. cerevisiae*, encoding a component of APC (27). To examine the genetic interaction between the two genes, UbcP4 was overproduced in *cut9*^{ts} mutant cells.

The UbcP4 cDNA cloned into pREP1, pREP41, and pREP81 expression vectors and cDNAs for UbcP1 (the *S. pombe* homolog of Ubc4 in *S. cerevisiae*) and UbcP2 (Ubc2/Rad6 homolog) cloned in pREP41 were transformed into cells of the *cut9*^{ts} strain. The nmt1 promoter in pREP41 and pREP81 is a truncated form of that in pREP1 and downregulates the transcription activity by approximately 1 and 2 orders of magnitude, respectively (5, 35). At 25°C, colony formation of *cut9*^{ts} cells was not affected by either overproduction or repression of the Ubcs (Fig. 9, left-hand plates), except that overproduction of UbcP1 resulted in a reduced rate of cell growth. At 34°C, overexpression of UbcP4 under the control of pREP1 and pREP41 supported the growth of *cut9*^{ts} cells, but when repressed, it did not (Fig. 9, right plates). pREP81-promoted overproduction was not sufficient to suppress the *cut9*^{ts} mutation at this temperature. The suppression of the *cut9*^{ts} mutation by the overproduction of E2 was specific to UbcP4, since neither overproduced UbcP1 nor UbcP2 at the same level as that of UbcP4 allowed the growth of *cut9*^{ts} cells at nonpermissive temperatures (Fig. 9, right plates).

Cdc13 accumulates in UbcP4-depleted cells. Suppression of the *cut9*^{ts} mutation by overexpression of UbcP4 strongly suggested that UbcP4 mediated, in conjunction with APC, a ubiquitin pathway for M-phase-specific proteolysis of mitotic cyclin and the chromatid adhesion proteins. We therefore examined the stability of Cdc13 in the UbcP4-depleted cells. Expression of *ubcP4* in FO201 cells was repressed with thiamine as in Fig. 4.

The intracellular level of Cdc13 protein was determined by immunoblotting of the cell extracts prepared along the time course of the UbcP4 depletion (Fig. 10). The amount of Cdc2 kinase detected by anti-PSTAIRE was used as an internal control of the protein content in the extracts. After 14 h of repression of *ubcP4*, the level of Cdc13 began to increase, which was coincident with the appearance of arrested cells. After that, Cdc13 continued to accumulate, and even after 22 h, the amount of Cdc13 protein was still increasing. Thus, Cdc13 protein was stabilized in UbcP4-depleted cells. This result suggested that UbcP4 depletion brought about cell cycle arrest at the stage at which the Cdc13 degradation machinery was not yet activated or that the UbcP4 was responsible for Cdc13 degradation process.

G_2 arrest is dependent on the *chk1* checkpoint pathway. Since many cases of G_2 arrest in the cell cycle are due to the activation of the checkpoint machinery, which surveys DNA damage or replication, we analyzed whether the G_2 arrest due to depletion of UbcP4 was related to the *chk1* checkpoint pathway. Taking advantage of the linkage of the *chk1* and *ubcP4* genes, a haploid strain, FO301, in which both *chk1* and *ubcP4* were replaced with the *ura4*$^{+}$ gene, was constructed (Fig. 3), and UbcP4 was conditionally expressed from cDNA under the control of nmt1 on pREP81. The *chk1* gene is not essential for cell viability. The UbcP4 protein was depleted by inoculating cells in the medium with thiamine as in Fig. 4, and after 18 h the arrested cells were analyzed by DAPI staining, flow cytometry analysis, and measurement of cell size distribution. Cells which had normal cell size and were blocked in progression from metaphase through anaphase were observed as for FO201 cells. However, no elongated cells, neither G_2- nor metaphase-arrested cells, were found among the UbcP4-depleted FO301 cells (Fig. 6, right). Even after prolonged incubation (until 24 h) for UbcP4 depletion, the elongated cells did not appear in thiamine-repressed FO301 cells. Flow cytometry analysis also indicated the accumulation of elongated cells

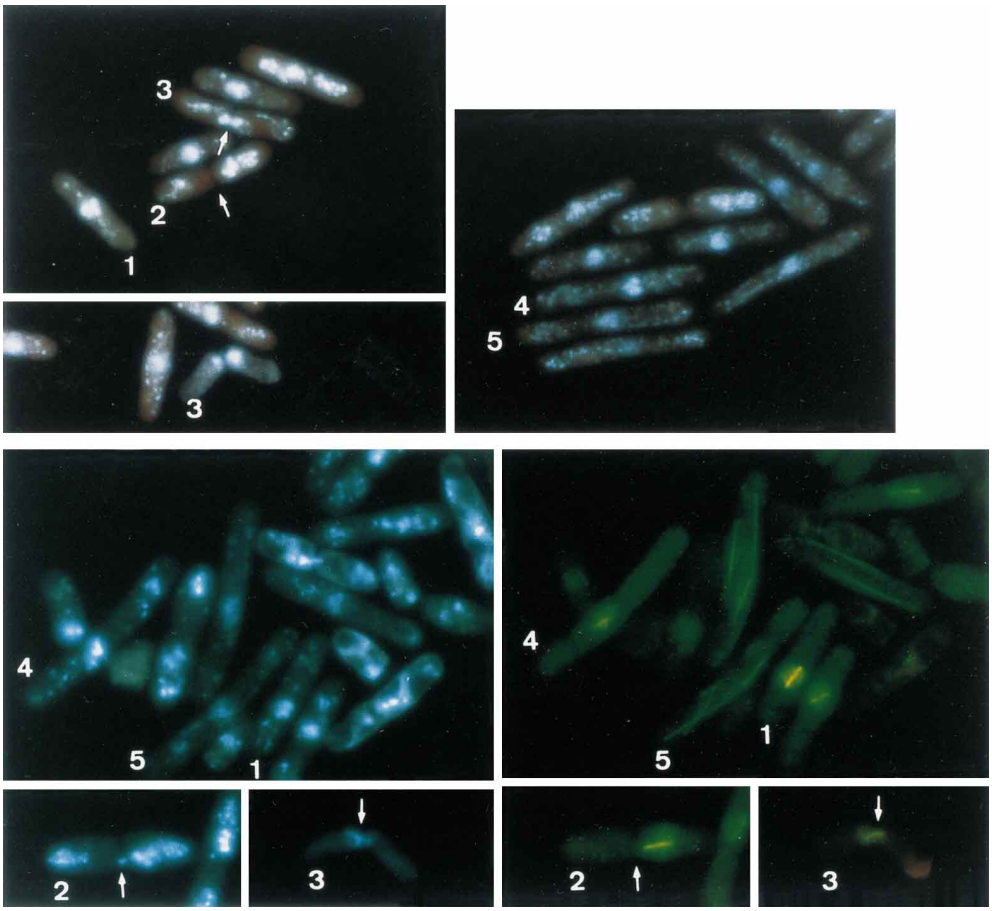

FIG. 5. Cell cycle phenotype of UbcP4-depleted cells. Cells stained with DAPI (upper and left of lower panels) or anti-tubulin antibody (right of lower panels). The left and right parts of the lower panels were from the same field. Examples of type A, B, C, D, and E cells are indicated by 1, 2, 3, 4, and 5, respectively.

in UbcP4-depleted FO201 cells but not in FO301 cells (data not shown). FO301 cells transformed with the *chk1*$^+$ gene cloned in a multicopy plasmid, pSP1, resulted in the reappearance of elongated cells which were arrested at G_2 or metaphase, after a 16-h incubation in the presence of thiamine (data not shown). Thus, G_2 arrest in UbcP4-depleted cells was completely dependent upon the *chk1* checkpoint pathway.

DISCUSSION

Most of the genes for E2 or Ubc have been surveyed, and at least 12 genes have been identified in budding yeast (24, 28). Many of them were isolated by the PCR method based on conserved sequences which had been found among a few basic *UBC* genes such as *UBC2/RAD6* (29) and *UBC3/CDC34* (10, 16, 41). Here, we screened cDNAs for E2 in fission yeast by a novel method which did not utilize sequence homologies but, rather, enzymatic activity. This enabled us to screen a structurally different class of E2 enzymes. Finally, we isolated 20 E2 clones in four types of genes. However, they all were included structurally in a single class of enzyme that has a conserved UBC domain common to all known E2s. The cDNA library we screened was neither normalized nor equalized, and the predominant isolates were those for UbcP1 and UbcP2, the *S. pombe* counterparts of Ubc4/5 and Ubc2/Rad6 in *S. cerevisiae*, respectively. Thus, UbcP1 and UbcP2 might be quantitatively the major E2s in *S. pombe* as Ubc4/5 and Ubc2/Rad6 are in *S. cerevisiae*.

Among the E2s so far identified, only two are known to be involved in cell cycle control. The *ubc3/cdc34* mutation caused cell cycle arrest at the G_1 phase (10, 16, 41), while a defect in *ubc9* did so at the G_2 and M phases (42) in *S. cerevisiae*. As discussed below, we showed that *ubcP4* was essential for both the G_2/M transition and the onset of anaphase in mitosis. *hus5* (2), a *S. pombe* homolog of *UBC9*, was distinct from *ubcP4* (Fig. 2). In addition to the UBC domain, UbcP4 contained an

TABLE 2. Time course and distribution of cell cycle phenotypes in UbcP4-depleted FO201 cells

Type[a]	Population (%) at following time (h) after thiamine addition:			
	0	14	16	18
A	<1	25	23	ND[b]
B and C	<1	19	28	ND
D[c]	<1	<1	6	18
E[c]	<1	<1	10	16

[a] Type A, metaphase-arrested cells with condensed chromosome; type B, cells with septum but not separated chromosome; type C, cells with mitotic catastrophe; type D, elongated cells with condensed chromosome; type E, elongated cells with decondensed chromosome.

[b] ND, not determined.

[c] Cells longer than 16 μm were counted as elongated. In the absence of thiamine, the typical cell length in the FO201 strain was 6 to 14 μm, which was the same as that of wild-type cells.

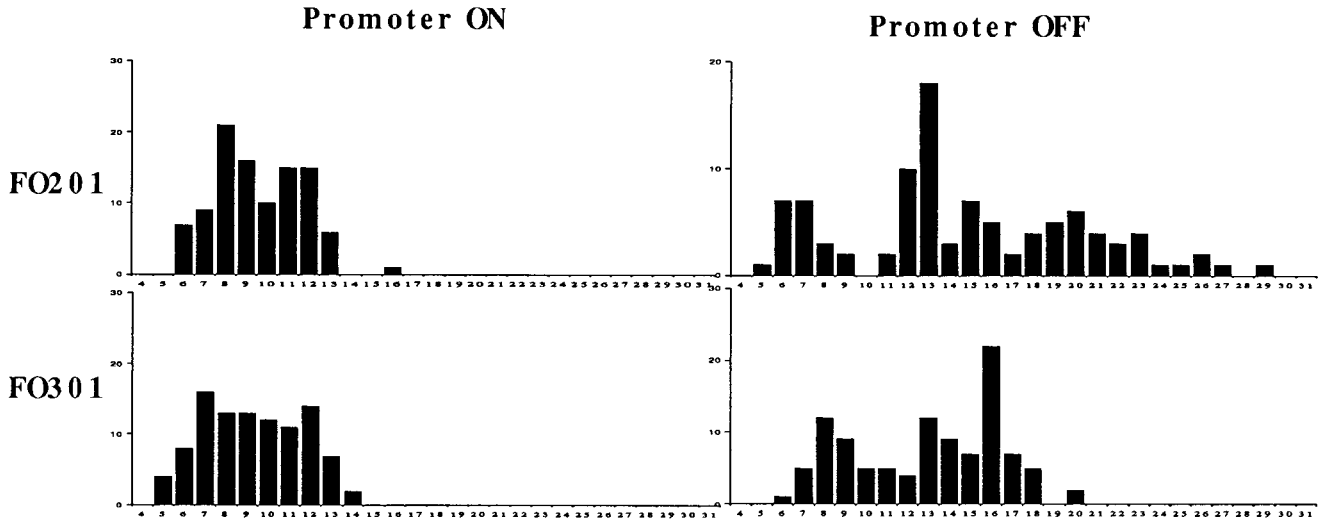

FIG. 6. Size distribution of UbcP4-depleted cells. Histogram of cell length (micrometers) measured in random pictures of FO201 (upper panels) or FO301 (lower panels) cells which were grown for 18 h in thiamine-free (left) and thiamine-containing (right) medium.

extension sequence in its N-terminal region, which distinguished this enzyme from other E2 enzymes. Thus, UbcP4 shares characteristic features in structure and function with neither Cdc34/Ubc3 nor Ubc9, indicating that it is a third case of E2 that is genetically defined to confer cell cycle functions. We showed here that UbcP4-depleted cells were defective at two stages of the cell cycle, G_2/M and metaphase/anaphase transitions. In metaphase-arrested cells, chromosomes were normally duplicated and condensed but could not be separated. Short spindles were formed but not elongated. Septum formation and cytokinesis were not restrained during the metaphase arrest by UbcP4 depletion. Thus, in UbcP4-depleted cells, the separation of sister chromatids was defective, preventing the onset of anaphase. These phenotypes resemble those observed in mutants of the series of *cut* genes in *S. pombe*.

The cells which were elongated and arrested at the G_2 phase emanated 2 h later after the appearance of metaphase-arrested cells. This does not necessarily mean that more restrictive depletion of the UbcP4 protein was required to cause G_2 rather than metaphase arrest in the cells. It would take G_2-arrested cells a somewhat longer interval to become elongated. The elongated cells arrested at metaphase with condensed chromosomes and short spindles (type D cells) appeared last. As for *cdc* mutants, elongated and metaphase-arrested cells such as D-type cells in this study, have not been reported so far. Furthermore, in a *chk1* mutant background, neither G_2- nor metaphase-arrested cells with an elongated shape were detectable. Thus, it is conceivable that the G_2 arrest of elongated cells (type E) somehow leaked through metaphase, resulting in type D cells.

UbcP4 may ubiquitinate a protein which confers functions at different stages, i.e., the G_2/M transition and the metaphase/anaphase transition, or two distinct proteins functioning at the two different cell cycle stages. The only case of similar phenotypes to those in the UbcP4-depleted cells, caused by a mutation in a single gene, was found in dominant negative mutants of Cdc2 kinase (12, 33). Their overproduction brings about a defect both in entering mitosis and in sister chromatid separation. Thus, the negative regulators of Cdc2 kinase might be targeted for degradation via UbcP4-mediated ubiquitination. Their prolonged stabilization by UbcP4 depletion may cause repression of Cdc2 kinase activity. This, however, was not the case because histone H1 kinase activity increased and was maintained at a high level in UbcP4-depleted cells.

Evidence that proteolysis of some proteins is essential for the progression of mitosis has accumulated. Abnormal mitosis occurs in cells of mutants with mutations in the subunits of the 26S proteasome (17, 18). *cdc* mutants such as *cdc27* and *cdc16* in *S. cerevisiae* showed metaphase arrest, and their products have been identified as components of 20S APC (27, 32, 44). APC is also required as a ubiquitin-ligase specific for the ubiquitination of mitotic cyclin. Although the destruction of mitotic cyclins is essential for the exit from mitosis, it is not responsible for the onset of anaphase in terms of sister chromatid separation. During the segregation, some other protein(s) such as Cut2 needs to be degraded (13). This is mediated by the same ubiquitin-ligase (APC) and proteasome pathway as for the mitotic cyclins (13, 25). The defect in sister chromatid separation in UbcP4-depleted cells was very similar to that in mutants of the cyclin-specific ubiquitination machinery. The *cut9* gene in *S. pombe* encodes a subunit of 20S APC. We showed that overexpression of the E2 protein, UbcP4, overcame the defect of the APC function as E3.

We showed that Cdc13 protein accumulated in UbcP4-depleted cells. This raised two possibilities. First, loss of the UbcP4 function might arrest cells at a cell cycle stage when mitotic cyclin degradation was not yet initiated. Alternatively, UbcP4 might be involved in the cyclin degradation machinery. We preferred the latter case. Metaphase-arrested cells formed a major fraction of the UbcP4-depleted cells. They were not restrained in mitosis and proceeded with cytokinesis, and hence the Cdc13 degradation machinery was expected to be activated in the metaphase-arrested cells. However, arrest at both the G_2 phase and metaphase occurred in cells lacking UbcP4. Therefore, we could not rule out the possibility that the Cdc13 protein accumulated in G_2-arrested cells but not in metaphase-arrested cells.

We observed that UbcP4 was required for sister chromatid separation. Overexpression of UbcP4 suppressed the APC defect. Cdc13 was stabilized in the cells lacking UbcP4. Taking these facts into consideration, together with the homology of

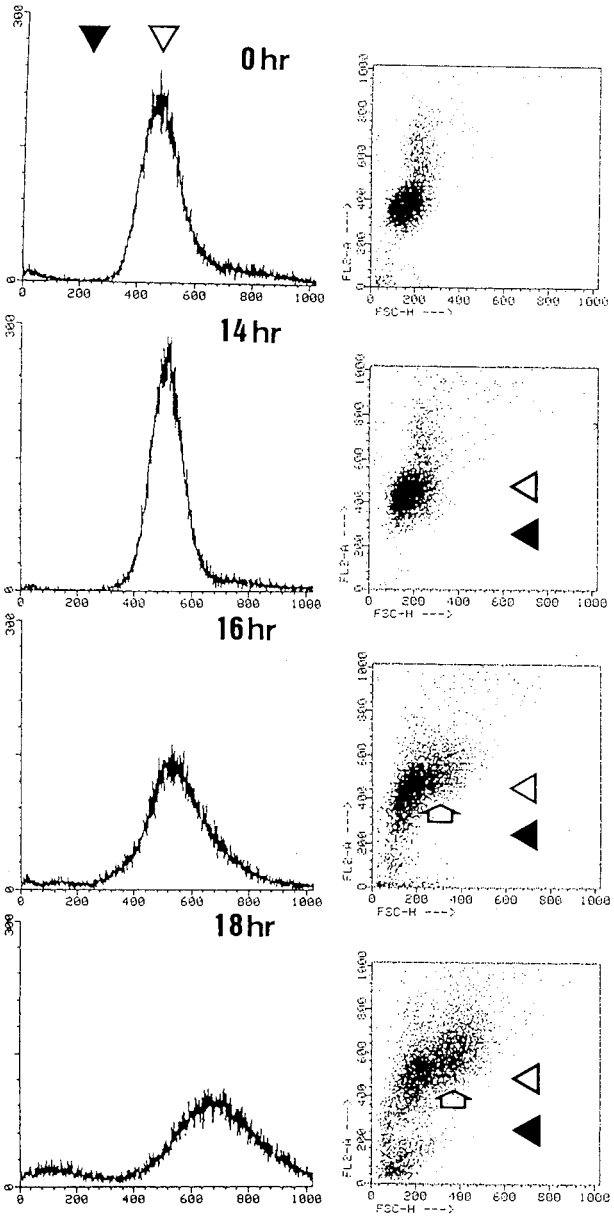

FIG. 7. Distribution of DNA content and size of UbcP4-depleted cells. FO201 cells repressed with thiamine as in Fig. 4 for the indicated periods were stained with propidium iodide and then subjected to flow cytometric analysis. Histograms of distribution of the DNA content are shown on the left, and dot plots with DNA content (vertical axis) and cell size (forward scattering; horizontal axis) of the same samples are shown on the right. Cell cycle-arrested cells usually show a slightly shifted DNA content, which is due to the increase in the amount extranuclear DNA. Open and solid triangles indicate the positions of 2C and 1C DNA content, respectively. The open arrows indicate the fraction of elongated cells.

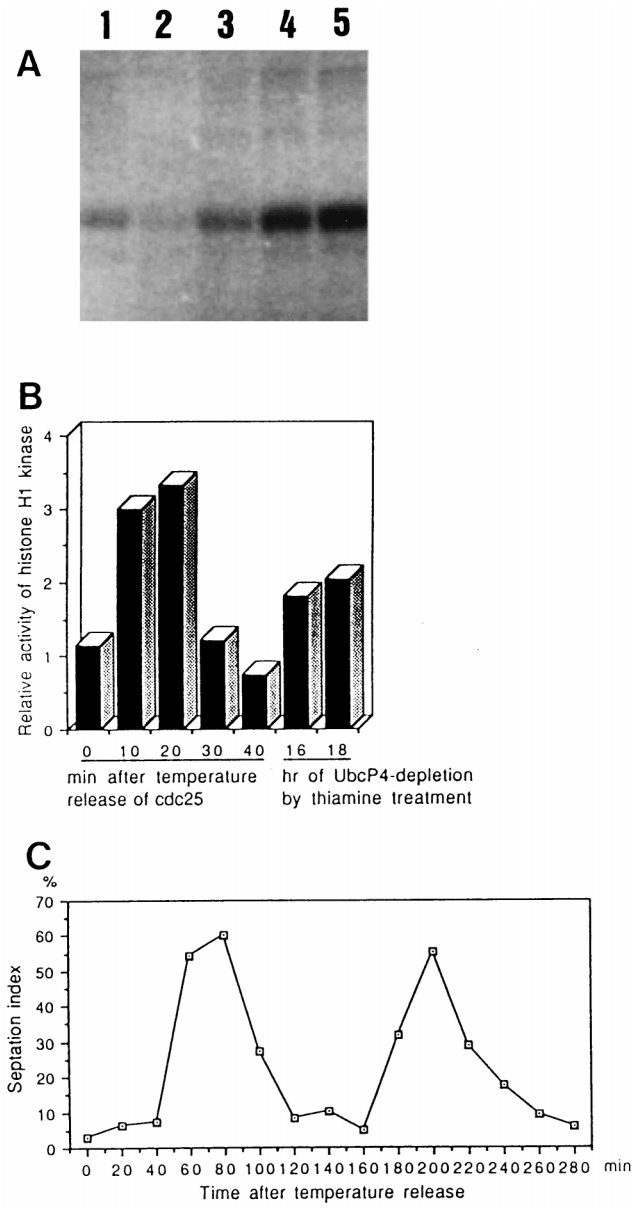

FIG. 8. Histone H1 kinase activity in UbcP4-depleted cells. (A) Histone H1 kinase activity in the lysates of FO201 cells repressed with thiamine for 0 h (lane 1), 5 h (lane 2), 12 h (lane 3), 14 h (lane 4), and 16 h (lane 5), as in Fig. 4, was assayed as described in Materials and Methods. (B) Comparison of histone H1 kinase activity in FO201 cells repressed with thiamine for 16 and 18 h with that in *cdc25*^{ts} cells synchronized at the G_2 phase by a temperature shift to 35°C for 5 h and then released by a shift down to 25°C for the indicated time. Radioactivity in the labeled histone H1 assayed as in panel A, was quantified and normalized with that from cells before the temperature release. (C) Synchronization of *cdc25*^{ts} cells was confirmed by the increase of the septation index.

UbcP4 to cyclin-specific E2s found recently in frog egg and clam extract (see below), we suppose that UbcP4 is a ubiquitin-conjugating enzyme working in conjunction with APC to mediate the ubiquitin pathway for degradation of "sister chromatid holding protein(s)" at the onset of anaphase and possibly of mitotic cyclin at the exit of mitosis. The UbcP4 homolog was widely distributed among lower and higher eukaryotes, implying its essential and universal function. Searches of the expressed sequence tag database identified a human UbcP4 homolog with more than 60% identity in amino acid sequence (T86566, R80990, and R95056). The full-length human homolog cDNA was isolated and shown to be highly homologous throughout the total sequence (unpublished results in collaboration with S. Kato, Sagami Chemical Research Center). A homolog in *S. cerevisiae* was found as an open reading frame with 66% identity in the genome sequence data on the right arm of chromosome XV (X95720), which, however, lacks the N-terminal segment specific to UbcP4. UbcP4 also shared 60%

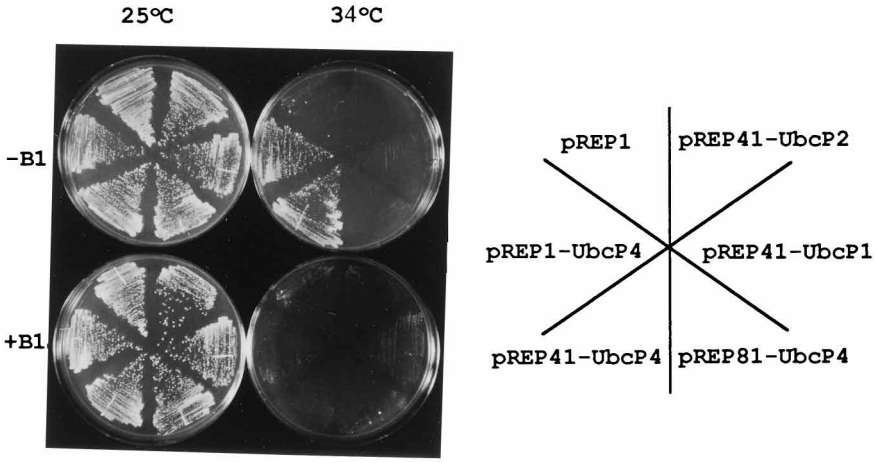

FIG. 9. Suppression of the *cut9*^{ts} mutation by UbcP4 overexpression. *cut9*^{ts} cells harboring the indicated plasmid were inoculated on MM agar with (lower plates) or without (upper) 10 μM thiamine and incubated at 25°C (left) or 34°C (right) for 6 days.

identity with *Xenopus* E2 UBC-x (48) and clam E2-c (3), which were recently reported to support in vitro ubiquitination of mitotic cyclin. We did not directly show the effects of UbcP4 on the ubiquitination of Cdc13, a mitotic cyclin in *S. pombe*. However, homology to the E2s responsible for cyclin B ubiquitination strongly suggests that UbcP4 might be involved in the ubiquitination of mitotic cyclin. Ubc9 in *S. cerevisiae* has previously been reported to play a role in the destruction of S- and M-phase cyclins, Clb5 and Clb2, respectively (42). UbcP4 was distinct from Hus5, a Ubc9 homolog in *S. pombe* (Fig. 2A). *hus5* is not essential for growth. The *hus5* mutant phenotype, in contrast to that of *ubcP4*, does not confer a clear defect in metaphase/anaphase transition (2). Furthermore, it was reported that the Ubc9 homolog protein in *Xenopus* egg extract does not copurify with cyclin ubiquitination activity (32, 48). Thus, UbcP4 is most likely to be an E2 responsible for cyclin ubiquitination in yeast. Two E2 enzymes in *Xenopus* egg extract, homologs of *S. cerevisiae* Ubc4 and UBC-x, have been shown to be involved in in vitro cyclin ubiquitination (32, 48). In fission yeast, *ubcP4*$^{+}$ is essential for the onset of anaphase. Overproduction of UbcP1, a homolog of *S. cerevisiae* Ubc4, did not suppress the *cut9*^{ts} mutation. These findings suggest that the UbcP1 pathway, if it is involved at all in chromosome segregation and cyclin destruction, is neither parallel to nor redundant with respect to the UbcP4 pathway in fission yeast.

The G_2 arrest of UbcP4-depleted cells was dependent on the Chk1-mediated checkpoint pathway. This suggests two possibilities. First, UbcP4 mediated the ubiquitination of a protein(s) functioning as a mediator or regulator of the checkpoint pathway upstream of Chk1. Second, UbcP4 depletion caused deficient or abnormal DNA synthesis, which activated the checkpoint pathway monitoring the DNA repair system. This implies that proteolysis of some proteins is required for the normal process of DNA synthesis. Very recently, it was reported that some mutant alleles of *CDC27* and *CDC16* caused rereplication of DNA without entering mitosis in *S. cerevisiae* (20). In this condition, cells exhibit G_2-like arrest, which is released through anaphase under a defect in the checkpoint pathway. We could not detect obvious rereplication of DNA with the repression of UbcP4. However, a possible idea is that the ubiquitin pathway, including UbcP4 and at least some components of APC, is concerned with the control of DNA replication by degrading proteins involved in the replication machinery.

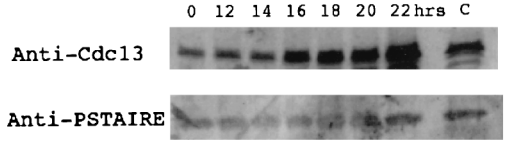

FIG. 10. Accumulation of Cdc13 in UbcP4-depleted cells. Extracts of FO201 cells repressed with thiamine as in Fig. 4 for the indicated periods were subjected to Western blotting with anti-Cdc13 (upper) and anti-PSTAIRE (lower) antibodies. C indicates extract from asynchronously growing cells.

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