The *Saccharomyces cerevisiae* Checkpoint Gene *BUB1* Encodes a Novel Protein Kinase

B. TIBOR ROBERTS,[†] KATIE A. FARR, AND M. ANDREW HOYT*

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218-2685

Received 20 May 1994/Returned for modification 14 July 1994/Accepted 15 September 1994

Normal cell multiplication requires that the events of mitosis occur in a carefully ordered fashion. Cells employ checkpoints to prevent cycle progression until some prerequisite step has been completed. To explore the mechanisms of checkpoint enforcement, we previously screened for mutants of *Saccharomyces cerevisiae* which are unable to recover from a transient treatment with a benzimidazole-related microtubule inhibitor because they fail to inhibit subsequent cell cycle steps. Two of the identified genes, *BUB2* and *BUB3*, have been cloned and described (M. A. Hoyt, L. Totis, and B. T. Roberts, Cell 66:507-517, 1991). Here we present the characterization of the *BUB1* gene and its product. Genetic evidence was obtained suggesting that Bub1 and Bub3 are mutually dependent for function, and immunoprecipitation experiments demonstrated a physical association between the two. Sequence analysis of *BUB1* revealed a domain with similarity to protein kinases. In vitro experiments confirmed that Bub1 possesses kinase activity; Bub1 was able to autophosphorylate and to catalyze phosphorylation of Bub3. In addition, overproduced Bub1 was found to localize to the cell nucleus.

The production of two viable and equivalent daughter cells in mitosis requires that the events leading to cell division proceed in a carefully ordered fashion. Among other tightly regulated events, replicated sister chromosomes must be properly segregated, one to each daughter cell. For this reason, mitosis cannot be allowed to proceed if the genome has not been fully replicated or if chromosomes are not properly attached to a fully assembled mitotic spindle. Mechanisms required for ensuring the dependency of cell division on completion of such prerequisite steps have been termed checkpoints (12). The first checkpoint-associated gene product described was Rad9 (37), which is required for delaying the cell cycle prior to mitosis for repair of damaged DNA. However, the biochemical activity of Rad9 and its precise role in enforcing normal cell cycle dependency relationships are still unclear.

In most eukaryotic cells, disruption of the spindle results in a cell cycle delay until the spindle is reassembled (5). Treatment of wild-type yeast cells with benzimidazole-related microtubule inhibitors leads to cell cycle arrest in mitosis. Each treated cell has a large bud and replicated DNA, and the cyclical kinase activity of M-phase promoting factor (MPF) is maintained in a highly elevated state (14, 19, 20). Inactivation of MPF and progression into subsequent phases of the cell cycle are dependent on the reassembly of microtubules.

We have previously described two mutants of the yeast *Saccharomyces cerevisiae*, designated *bub1* and *bub2* mutants, which are unable to delay the cell cycle in response to loss of microtubules (14). A third gene, *BUB3*, was identified as an extra-copy suppressor of the *bub1-1* mutant. Like the *bub1* and *bub2* mutants, *bub3* deletion strains (*bub3Δ*) do not arrest at G₂/M when treated with large doses of benzimidazole but instead continue to bud and rapidly lose viability. Three mutations with similar phenotypes, designated *mad1*, *mad2*,

and *mad3*, have been described elsewhere (20). None of these are allelic to the *bub* mutations (11, 13a).

BUB2 and *BUB3* have been cloned and sequenced (14). Bub2 is closely related to the *cdc16⁺* gene product of *Schizosaccharomyces pombe* (7). The primary sequence of Bub3 is unrelated to sequences of other known proteins (14). Neither gene is essential, although *bub3* mutants grow very slowly. Here we present the cloning and characterization of *BUB1*, which encodes a gene product with a carboxyl-terminal domain similar to those of protein kinases. We demonstrate that Bub1 autophosphorylates in vitro and provide genetic and immunological evidence that Bub3 physically interacts with and is a substrate for the Bub1 kinase. We show that when overexpressed together, these proteins interfere with colony formation. Finally, we demonstrate that overproduced Bub1 is directed to the nucleus.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in these experiments were derivatives of S288C and are listed in Table 1. The screen which yielded the *bub1-1* mutation has been described, along with the construction of the *bub3Δ::LEU2* allele (14). Rich (YPD), minimal (SD), and sporulation media were as described elsewhere (30). The benzimidazole compound benomyl (DuPont) was added to solid media from a 10-mg/ml stock in dimethyl sulfoxide. Concentrations to assess benomyl sensitivity were in the range of 5 to 15 μg/ml. For overexpression in galactose liquid medium, strains were transferred from fresh patches on SD lacking uracil to liquid synthetic medium lacking uracil with 2% raffinose (Sigma) as the sole carbon source. After 24 to 36 h at 26°C, rapidly growing cultures were diluted to an optical density at 600 nm of 0.03, and galactose (Sigma G-0750) was added to 2%. Cultures were grown 12 to 14 h longer and then harvested. For galactose-induced overexpression on plates, fresh patches of strains on SD plates were transferred to solid synthetic medium lacking uracil with raffinose at 2% and grown for 30 h at 30°C. These strains were then transferred to synthetic agar medium with 2% galactose with various concentrations of benomyl and photographed after 3 days at 26°C. To examine

* Corresponding author. Mailing address: Department of Biology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218-2685. Phone: (410) 516-7299. Fax: (410) 516-5213. Electronic mail address: hoyt_ma@jhvmhs.hcf.jhu.edu.

[†] Present address: Howard Hughes Medical Institute, University of Colorado School of Medicine, Denver, CO 80262.

TABLE 1. Yeast strains used

Strain	Relevant genotype
MAY589	<i>MATa BUB⁺ ura3-52</i>
MAY591	<i>MATα BUB⁺ ura3-52</i>
MAY1210	<i>MATa/MATα BUB⁺/BUB⁺</i>
MAY1800	<i>MATa bub1-1 ura3-52</i> (pMA1145 = <i>BUB3</i> , <i>URA3</i> , <i>CEN</i>)
MAY2072	<i>MATa bub3Δ::LEU2 ura3-52</i>
MAY2150	<i>MATa bub1-1 trp1-Δ1</i>
MAY2352	<i>MATa bub1-1 trp1-Δ1</i> (pTR37 = <i>BUB1</i> , <i>TRP1</i> , <i>CEN</i>)
MAY2409	<i>MATα BUB1::URA3::BUB1</i>
MAY2517	<i>MATa/MATα bub1Δ::HIS3/BUB1</i>
MAY2589	<i>MATa bub1Δ::HIS3</i>
MAY2740	<i>MATa/MATα bub1Δ::HIS3/BUB1 bub3Δ::LEU2/BUB3</i>
MAY3188	<i>MATa BUB⁺ ura3-52</i> (pTR144 = pGAL- <i>BUB1</i> , pGAL- <i>BUB3</i> , <i>URA3</i> , 2 μ m)
MAY3275	<i>MATa BUB⁺ ura3-52</i> (pTR147 = pGAL- <i>bub1K733R</i> , pGAL- <i>BUB3</i> , <i>URA3</i> , 2 μ m)
MAY3316	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR143 = pGAL- <i>BUB1</i> , <i>URA3</i> , 2 μ m)
MAY3317	<i>MATa BUB⁺ ura3-52</i> (YE352 = <i>URA3</i> , 2 μ m)
MAY3335	<i>MATa BUB⁺ ura3-52</i> (pTR143 = pGAL- <i>BUB1</i> , <i>URA3</i> , 2 μ m)
MAY3336	<i>MATa/MATα bub1Δ::URA3/BUB1</i>
MAY3337	<i>MATa bub1Δ::HIS3 ura3-52</i> (YE352 = <i>URA3</i> , 2 μ m)
MAY3339	<i>MATa bub1Δ::HIS3 ura3-52</i> (pTR150 = pGAL- <i>BUB3</i> , <i>URA3</i> , 2 μ m)
MAY3340	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR150 = pGAL- <i>BUB3</i> , <i>URA3</i> , 2 μ m)
MAY3341	<i>MATa BUB⁺ ura3-52</i> (pTR150 = pGAL- <i>BUB3</i> , <i>URA3</i> , 2 μ m)
MAY3343	<i>MATa bub3Δ::LEU2 ura3-52</i> (YE352 = <i>URA3</i> , 2 μ m)
MAY3419	<i>MATa bub1-1 ura3-52</i> (YE352 = <i>URA3</i> , 2 μ m)
MAY3420	<i>MATa bub1Δ::HIS3 ura3-52</i> (pMA1145 = <i>BUB3</i> , <i>URA3</i> , <i>CEN</i>)
MAY3421	<i>MATa bub1-1 ura3-52</i> (pTR143 = pGAL- <i>BUB1</i> , <i>URA3</i> , 2 μ m)
MAY3422	<i>MATa bub1-1 ura3-52</i> (pTR148 = pGAL- <i>bub1K733R</i> , <i>URA3</i> , 2 μ m)
MAY3423	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR148 = pGAL- <i>bub1K733R</i> , <i>URA3</i> , 2 μ m)
MAY3594	<i>MATa bub1Δ::URA3</i>
MAY3598	<i>MATa bub3Δ::LEU2</i>
DK4795-411	<i>MATa ade3</i>

inhibition of budding by microtubule disruption, *MATa* cells were arrested in G₁ with α -factor, released onto benomyl (70 μ g/ml)-containing solid medium for 6 h, and observed microscopically as described previously (14).

DNA manipulations. A *TRP1-CEN* plasmid library of *S. cerevisiae* DNA (4a) was transformed into a *bub1-1 trp1- Δ 1* strain (MAY2150). *Trp⁺* transformants were replica transferred to YPD plates containing 10 μ g of benomyl per ml. Resistant clones were then screened for loss of the *bub* continued-budding-in-benomyl phenotype as described previously (14). Plasmids from benomyl-resistant (Ben^R) colonies were extracted and transformed into *Escherichia coli* for analysis. Subcloning of the original genomic inserts to establish the boundaries of the genes was done by standard procedures. The cloned inserts could represent either the actual *BUB1* locus or a suppressor locus. To distinguish between these possibilities, the *ClaI-SacI* region of the genomic insert from pTR37 was subcloned into the yeast integrating vector pRS306 to generate pTR50. This construct was cut with *XbaI* and transformed into MAY591. The resulting strain, which was *BUB⁺*, was mated to the *bub1-1* strain, MAY2150. When sporulated, this diploid gave rise to 16 parental ditype asci and no nonparental or tetratype asci.

Marked disruptions of *BUB1* were generated by the one-step gene replacement method (28). A 2.5-kb DNA fragment containing *HIS3* was inserted between the *XbaI* and *SmaI* sites in *BUB1* in pTR50, resulting in the deletion of 942 bp of the wild-type gene in pTR65. A 6.7-kb *EcoRV-SacI* fragment from this plasmid encompassing the deletion was used to transform the diploid strain MAY1210, yielding the *bub1 Δ ::HIS3* heterozygote MAY2517. The resulting transformants were sporulated to generate haploid null spores. Proper insertion was confirmed by Southern analysis using a radioactively labeled

2.6-kb *HpaI-PvuII* fragment of *BUB1* (data not shown). This same *BUB1* probe hybridized to yeast genomic DNA lambda library clone 4572 (data not shown) derived from chromosome VII (27a). Viability of putative *bub1* null mutants was later confirmed by creation of a more complete deletion of *BUB1*. A 2.5-kb fragment between the outermost *HindIII* sites, including all of the sequence with significant homology to known protein kinases, was replaced with *URA3* to make pTR92. A diploid strain heterozygous for *bub1 Δ ::URA3* (MAY3336) was generated by linearizing pTR92 with *SalI* and *BamHI* and transforming MAY1210 as described above. Survival and benomyl sensitivity of spores from the *bub1 Δ ::URA3* and *bub1 Δ ::HIS3* heterozygotes appeared identical.

Viability of *bub1 Δ ::HIS3 bub3 Δ ::LEU2* double-disruption mutants was established by transformation of the *bub3 Δ ::LEU2/BUB3* diploid strain MAY2067 (14) with *bub1 Δ ::HIS3* as described above to make MAY2740, a double heterozygote. This diploid was sporulated and analyzed as described above. Identical results were obtained by transforming MAY2517 (*bub1 Δ ::HIS3/BUB1*) with *StuI-SacI*-linearized pTR27 (*bub3 Δ ::LEU2*) (14) to yield MAY2739.

pTR168, a *CEN* plasmid marked with *LEU2* and that expresses *BUB1* from its normal promoter, was constructed by subcloning the *SalI-BamHI* fragment from pTR37 into pRS315 (31). Overexpression of *BUB1* under the control of a galactose-inducible promoter was accomplished by inserting *BamHI* linkers at the *HpaI* site 24 bp 5' of the *BUB1* start codon and then transferring the resulting 3.6-kb *BamHI* fragment to the *BamHI* site of pBM272 (a gift from M. Johnston), yielding pTR134. A 4.6-kb *SalI-EcoRI* fragment from pTR134 including *BUB1* under the control of the *GAL1* promoter plus the linked *GAL10* promoter was transferred to the *URA3*-2 μ m plasmid YE352 (13), generating pTR143. *EcoRI* linkers were

ligated to a 1.1-kb *EcoRV* fragment beginning 31 bp upstream of the start ATG and ending 1 bp downstream of the stop TGA of *BUB3*. This fragment was inserted in the proper orientation for expression from the *GAL10* promoter at the *EcoRI* site of pTR143; this arrangement yields pTR144, a plasmid which overexpresses both proteins when grown in galactose medium. Overproduction of Bub3 alone was achieved by eliminating the *BUB1*-containing *BamHI* fragment from pTR144 to yield pTR150.

Construction of the *bub1K733R* allele was accomplished by site-directed mutagenesis (16, 17), using the synthetic oligonucleotide TO862 (GGACATCTGCGAGCGTTGAGAGTAG AAAAACCCTGCAACT) as a primer. A small (251-bp) *PacI*-*BstI* 1071 fragment of the resulting allele, determined by DNA sequencing analysis to have only the single base pair substitution responsible for the *bub1K733R* mutation, was subcloned into the wild-type *BUB1* gene for expression. pTR170 is a *CEN* plasmid that expresses this mutant *BUB1* from its normal promoter and is otherwise completely identical to pTR168 containing wild-type *BUB1* (see above). Overexpression of *bub1K733R* along with *BUB3* was accomplished by replacing 251 bp encoding part of the presumed Bub1 ATP binding site in pTR144 with the mutated region described above, generating pTR147.

DNA sequencing was performed with the Sequenase reagent kit (United States Biochemical) following subcloning into M13-based vectors or by using double-stranded templates. All reported sequence was determined from both strands of DNA.

Production of antibodies. The 1.4-kb portion of *BUB1* between the two amino-terminal *HindIII* sites was expressed in *E. coli* BL21 from the pRSETC vector (Invitrogen) as pTR124. This arrangement adds 47 amino acids at the amino terminus, including a six-adjacent-histidine motif, and 20 amino acids at the carboxyl terminus. A 200-ml culture in log phase was induced to express the fusion protein with 1 mM isopropylthiogalactopyranoside (IPTG) and then harvested. Cells were lysed by heavy sonication in buffer A (100 mM sodium phosphate, 10 mM Tris, 8 M urea [pH 8.0]). After spinning out insoluble debris, this sample was loaded three times over Ni^{2+} -nitrilotriacetic acid-agarose (Qiagen). The column was washed with 3 volumes of buffer A and 3 volumes of buffer B (buffer A, pH to 6.3). Finally, samples were eluted with buffer C (buffer A, pH to 5.9). Fractions containing the largest amounts of the fusion protein were pooled and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). The major band, with an apparent molecular mass of 64 kDa as visualized by copper chloride staining, was excised and then pulverized. Immunizations of two New Zealand White rabbits, designated 1026 and 1029, were performed by HRP Inc. (Denver, Pa.) by standard methods. Serum from rabbit 1026 was used in all experiments described herein because it had a higher affinity for Bub1 than that from rabbit 1029. Serum from the fourth bleed of this rabbit, designated 1026-4, was used in these experiments. Where noted, these antibodies were purified by the method of Olmsted (21) as described by Pringle et al. (27), using the 64-kDa fusion protein from pTR124 described above. Whole cell lysate from a 500-ml culture of cells expressing the 64-kDa protein was separated by preparative SDS-PAGE for transfer to nitrocellulose. The major band as visualized by Ponceau S staining was excised and treated exactly as described previously (27). Following elution and neutralization, antibodies were concentrated to approximately serum concentration in phosphate-buffered saline (PBS), using a Centricon (Amicon) spin filter system (molecular mass cutoff of 10^4 kDa).

Bub3-specific antibodies were a generous gift from Suzanne Guénette and Frank Solomon.

Immunofluorescence. Cells overexpressing *BUB* genes as described above were sonicated briefly and then fixed for 2 h in 3.7% formaldehyde at room temperature. Fixed cells were prepared for immunofluorescence microscopy as described previously (27) and incubated overnight at 4°C in 1:50-diluted affinity-pure 1026-4 primary antibodies (described above) and 1 h at room temperature with 1:250-diluted fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies.

Immunoprecipitation and autophosphorylation analysis. Immunoprecipitations were performed with affinity-purified antibodies from the fourth bleed of rabbit 1026 (1026-4) or preimmune antibodies from the same rabbit subjected to the same affinity purification regimen. In each case, a quantity of affinity-pure antibodies corresponding to 20 μl of untreated serum was used to immunoprecipitate Bub1 from 300 to 360 μg of soluble yeast protein. These protein extracts were obtained from strains described above by bead beating in the cold with PBS plus a freshly added protease inhibitor cocktail including leupeptin (0.5 $\mu\text{g}/\text{ml}$), chymostatin (0.1 $\mu\text{g}/\text{ml}$), aprotinin (2 $\mu\text{g}/\text{ml}$), and antipain (2.5 $\mu\text{g}/\text{ml}$) from a 100 $\times$ stock in water, plus pepstatin A (1 $\mu\text{g}/\text{ml}$) and phenylmethylsulfonyl fluoride (10 $\mu\text{g}/\text{ml}$) from a 100 $\times$ stock in ethanol. Antibody incubations were performed at 4°C for 3 to 5 h in PBS plus protease inhibitors (PBSPI). Immune complexes were collected by incubation at 4°C for 0.5 to 1.5 h with 80 μl of 50% protein A-Sepharose (Sigma P-3391) in PBS plus 0.1% bovine serum albumin (BSA) plus protease inhibitors (PBSBPI). Immune complexes were washed at least six times with 10 volumes of PBSBPI. An aliquot was washed at least two additional times with 10 volumes of PBSPI (no BSA) and, after boiling with 2 $\times$ sample buffer, reserved for Western blot (immunoblot) analysis. The remainder was treated essentially as described previously (4). Briefly, immune complexes were washed twice in 10 volumes of autophosphorylation buffer (50 mM Tris [pH 7.5], 10 mM MgCl_2 , 1% Triton X-100 [Sigma]). The volume was reduced to 30 μl , and 30 μCi of [γ - ^{32}P]ATP (Amersham) was added to each. The reaction was allowed to proceed 15 min at room temperature and was then terminated by addition of 2 $\times$ sample buffer and boiling. After SDS-PAGE, Coomassie blue staining, and destaining in 10% methanol-7% acetic acid-10 mM sodium pyrophosphate, radioactive bands were identified by autoradiography.

Western transfers to nitrocellulose (Schleicher & Schuell) to assess *BUB1* and *BUB3* overexpression and efficiency of immunoprecipitation were performed by standard methods (36). Proteins were visualized by using the Western Light (Tropix) chemiluminescence detection kit, with the modification that 5% powdered milk was added to the blocking agent, and blots were blocked overnight at 4°C. Except where noted, Western blots were probed with unpurified antiserum diluted 500- to 1,000-fold and incubated for 1 to 2 h at room temperature or overnight at 4°C.

Nucleotide sequence accession number. The sequence of the *BUB1* gene has been submitted to GenBank and assigned accession number L32027.

RESULTS

Cloning of *BUB1*. *BUB1* was cloned by transformation of the *bub1-1* strain MAY2150 with a yeast genomic library. Transformants were replica transferred to media containing 10 μg of benomyl (a benzimidazole compound) per ml, and resistant colonies were selected. Plasmids from the Ben^R colonies were isolated, retransformed into the original *bub1-1* strain, and

FIG. 1. Restriction enzyme site map of the *BUB1* locus. Thickened regions of the map indicate the extent of the open reading frame, with the arrowhead directed toward the 3' end. Lines beneath the restriction maps indicate DNA that is contained within tested subclones. + or - indicates whether the subclone rescues *bub1-1*. Restriction enzyme sites: Sl, *SalI*; Xh, *XhoI*; R, *EcoRI*; H, *HindIII*; X, *XbaI*; Sm, *SmaI*; P, *PstI*; S, *SacI*.

then tested again for ability to restore wild-type benomyl resistance. Those which invariably yielded Ben^R colonies were selected for further study.

Since *BUB3* is an unlinked, extra-copy suppressor of *bub1-1* (14) and extra copies of α -tubulin genes have been shown to increase benomyl resistance in wild-type cells (29), the restriction enzyme maps of genomic clones obtained by the screen were compared with the maps of *BUB3* and the yeast α -tubulin genes, *TUB1* and *TUB3*. One plasmid, pTR37, had a distinct restriction site pattern. The cloned region was marked with *URA3*, using a yeast integrating vector in a *ura3-52 BUB*⁺ haploid strain. The resulting strain, which was also *BUB*⁺, was mated to a *ura3-52 bub1-1* strain and sporulated. The spores were scored for benomyl resistance and uracil prototrophy. All 16 tetrads tested were parental ditypes with respect to these phenotypes (2 Ura⁺ Ben^R spores:2 Ura⁻ Ben^S spores). Among three tested tetrads, all Ura⁻ spores continued to bud on high concentrations of benomyl, and all Ura⁺ spores arrested normally. Taken together, these results indicate a tight linkage between *bub1-1* (≤ 8 centimorgans) and the introduced *URA3* marker and suggest that pTR37 includes the wild-type *BUB1* gene.

A radioactively labeled fragment of the *BUB1* gene was used to probe a Southern blot of *S. cerevisiae* chromosomes separated by pulsed-field gel electrophoresis. This experiment indicated that *BUB1* is located on chromosome VII. Hybridization of the same fragment to a lambda library of yeast genomic DNA fragments demonstrated linkage to *ADE3* (see Materials and Methods). This linkage was confirmed by tetrad analysis of a diploid heterozygous for *ade3* and a *URA3*-marked allele of *BUB1*. This experiment indicated that *BUB1* and *ADE3* are located approximately 12 centimorgans apart (39 parental ditype: 0 nonparental ditype: 12 tetratype).

Disruption of *BUB1*. Subcloning of DNA from pTR37 indicated that rescue of the *bub1-1* phenotype was conferred by a region contained between the *EcoRI* and *SacI* sites located 3.7 kb apart (Fig. 1). This region was sequenced and shown to contain a single 3,063-bp open reading frame (Fig. 2). Marked disruptions of *BUB1* were generated by the one-step gene replacement method (28) (see Materials and Methods).

bub1::HIS3 deleted DNA between the *XbaI* and *SmaI* sites internal to the *BUB1* gene (Fig. 1). *bub1 Δ ::URA3* deleted all of the DNA between the outermost *HindIII* sites in the *BUB1* gene, causing elimination of almost the entire open reading frame, including all sequences encoding protein kinase similarities (see below). Both *BUB1* disruptions caused identical phenotypes. Transformation of a wild-type diploid strain with these constructs yielded *bub1 Δ /BUB1* heterozygotes (MAY 2517 and MAY3336). When sporulated, the *bub1 Δ* spores germinated with reduced efficiency (Fig. 3); for MAY3336, 66% (76 of 116) of Ura⁺ spores failed to form colonies, while only 4% (5 of 116) of Ura⁻ spores failed. The viable *bub1 Δ* spores formed colonies that grew at much slower rates than their *BUB1* sisters (Fig. 3). When propagated, this slow-growth phenotype partially reverted, probably reflecting the accumulation of suppressing mutations. *bub1 Δ* cells were also super-sensitive to benomyl (dead at 7.5 μ g/ml, compared with 15 μ g/ml for the wild type; see Fig. 5). Similar phenotypes were reported for *bub3 Δ* mutants (14) although *bub3 Δ* spores germinated with wild-type efficiency.

bub deletion mutant cells were examined for the ability to halt their budding cycles when microtubule function was compromised (Table 2). Mutant and wild-type cells were synchronized in G₁ with α -factor and released onto medium containing a high concentration of benomyl. After 6 h, the *BUB*⁺ cells had stopped budding and arrested with a large-budded morphology (equivalent to two cell bodies in Table 2). In contrast, like the *bub1-1* mutant (14), both the *bub1 Δ* and *bub3 Δ* mutants frequently produced extra buds in the presence of benomyl.

The predicted *BUB1* gene product has similarity to known protein kinases. The predicted 1,021-amino-acid, 118-kDa protein encoded by the *BUB1* gene (Fig. 2) was compared with proteins in the GenPept database, using the FASTA and BLAST programs (1, 25). A carboxyl-terminal portion displays significant similarity to known serine-threonine protein kinases (Fig. 4). In particular, Bub1 has strong homology to kinases in the regions designated I, II, III, VI, and VII and weak homology in region IX (10). Note that the highly conserved regions VIII and XI are not found in Bub1, and there is an

```

-100 TACTACACAATATACGTGATTTTGAATAGAGTATAAAGTTTTCATCATGGAAGATTATTGACGGTTCCTATTGTTTGAATGTTAACGCTGACCAGGAA
1 M N L D L G S T V R G Y E S D K D T F P Q S K G V S S S S Q K E Q H S
1 ATGAATTTAGATTAGTTTCTACTGTACGAGGCTATGAATCAGATAAAGACACATTTCGCAATCGAAAGCGGTAGCTCATCTCAAAAAGAGCAGCAT
35 Q L N Q T K I A Y E Q R L L N D L E D M D D P L D L F L D Y M I W
101 GTCAACTGAATCAGACAAAATAGCTTATGAACAGAGACTTCTAAATGATTAGAGGATATGGATGATCCTTTAGATTGTTTGTATATATGATATG
68 I S T S Y I E V D S E S G Q E V L R S T M E R C L I Y I Q D M E T
201 GATTAGCACCAGCTATATTGAAGTAGATTCTGAATCAGGTCAAGAGTCTCCGAGTACAATGAAAGATGCTTGATTACATACAGATATGGAACCC
101 Y R N D P R F L K I W I W Y I N L F L S N N F H E S E N T F K Y M F
301 TATAGGACGATCCAGATTTTAAAAATTTGGATTGGTATATAAATTTGTTTCTTAGCAACAACCTCCAGGAGAGTGAATACTTTTAAATATATGT
135 N K G I G T K L S L F Y E E F S K L L E N A Q F F L E A K V L L E
401 TTAACAAGGGAATCGGAACAAAGCTTTCGTTATTTATGAAGAGTCTCCAAGCTACTAGAAAATGCTCAATTTTTCTAGAAGCAAGGTGTTATTAGA
168 L G A E N N C R P Y N R L L R S L S N Y E D R L R E M N I V E N Q
501 ACTAGGGCTGAAACCAATTCGAGGCTTACACAGACTATTAAGGTCAATTAAGCAATTATGAAGACAGACTCCGAGAAATGAACATCGTAGAAAACAG
201 N S V P D S R E R L K G R L I Y R T A P F F I R K F L T S S L M T D
601 AACAGTACCTGATTCAAGGGAAGATTGAAGGAGGTAAATTTATGAACAGCACCATTTCCTCGTAAATTTTAACTCATCTGATGATGACTG
235 D K E N R A N L N S N V G V G K S A P N V Y Q D S I V V A D F K S
701 ATGACAAGGAAATCGTGCAGCTGAACCTCTAATGTTGGAGTTGGTAAGTCTGCTCTAAGTATATCAAGATTTCTAGTGGTCTGCTGACTTAAAGTC
268 E T E R L N L N S S K Q P S N Q R L K N G N K K T S I Y A D K Q K
801 AGAAGCAGAGAGGCTTAATTTGAATAGCTTAAACAGCCCTCTAACCAACGCTTGAAGAATGAAATAAAAACTCAATATATGAGATCAAAAGCAA
301 S N N P V I G T K L I N T P G R K P E R I V F N F N L I Y P N D E E F
901 TCTAACATCCAGTCTACAACTAATCAATACTCCGGGACGGAACCTGAACGGATAGTTTTAATTTTAATCTAATATATCCAGAAAATGATGAGGAGT
335 N T E E I L A M I K G L Y K V Q R R G K K H T E D Y T S D K N R K
1001 TCAACCCAGGAAATATTTGCAATGATTAAAGGACTTACAAGTCAAGCAGGAGGTAAGAAGCACACCCGAGATTAACAAGTACGAAATAGGAA
368 K R K L D V L V E R R Q D L P S S Q P P V V P K S T R I E V F K D
1101 GAAACGTAAGCTCGATGTGCTGTAGAGAGCGCAAGACTTACCTTCATCACAGCCACCGGTGTCCTCCCAATCTACGCGAATGAGGTGTTAAGAT
401 D D N P S Q S T H H K N T Q V Q V Q T T S I L P L K P V V D G N L
1201 GACGATATCCAGGCGAGCAGCACACCATAAAAATACACAGGTACAAGTGCAACGCAACACGCTCTATTTACCTTTGAAGCGTGTGGTTGATGGAACCC
435 A H E T P V K P S L T S N A S R S P T V T A F S K D A I N E V F
1301 TCGCGATGAGACACAGTGAAGCCCTCACTAACATCCAAGCTTCCCGTTACCTACAGTAACAGCTTTTCAAAGACGCTATAAATGAGGTCTTTTC
468 M F N Q H Y S T P G A L L D G D T T T S K F N V F N L I Y P N D E E F
1401 CATGTTCAATCAACATTATTCAACCCCGGTGCGCTATTAGATGGTGATGATACCACTACAAGTAAATCAACGCTCTCGAAAATTTTACCAAGAATTT
501 T A K N I E D L T E V K D P K Q E T V S Q Q T T S T N E T N V R Y E
1501 ACAGCAAGAAATATTGAAGATTAACTGAAGTTAAAGATCTTAAACAGAGAGCGGTGTCAACAACTACCTCAACAAATGAACCAACGATACGATATG
535 R L S N S S T R P E K A D Y M T P I K E T T E T D V V P I I Q T P
1601 AAAGATTGTGCAATAGTACGACAGGCCAGAAAGCAGACTACATGACGCCCTATAAAGAACTACTGAAACAGATGTGGTACCTATAATCCAGACCC
568 K E Q I R T E D K K S G D N T E T Q T Q L T S T T I Q S S P F L T
1701 TAAGGAACAAATAGGACAGAAGATAAGAAATCGGGGATAATACAGAAACCAAACTCAATTAACAGCACAACCATCAAGTTCTCCATTCTCACA
601 Q P E P Q A E K L L Q T A E H S E K S K E H Y P T I I P P F K I K
1801 CAACCTGAACCAACGAGCAAAAGCTTTTCAAACTGCAGAACATTCGGAAGAGTAAAGAGCATTACCCAACGATCATCTCCCTTCACTAAATAA
635 N Q P P V I I E N P L S N N L R A K F L S E I S P P L F Q Y N T F
1901 AAAATCAACCGCTGTCTATTGAAAACCCACTTAGTAATAATTTAAGAGCGAAGTTTTATCCGAAATTTGCCCCCATTTTCAATATAACACCTT
668 Y N Y N Q E L K M S S L L K K I H R V S R N E N K N P I V D F K K
2001 TTATAACTACAACCAAGATTTGAAAATGAGTTCTCTATTGAAAAAATCCATAGGGTATCCCGCAACGAAACAAAGAACCCAAATTTGTGACTTCAAGAAG
701 T G D L Y C I R G E L G E G G Y A T V Y L A E S S Q G H L R A L K V
2101 ACGGTGATTATCTGATACGCGGAGAATTAGCGCAAGGAGTTTATGCCATGTATATCTAGCAGAATCTAGTCAAGGACATTCGAGCGGTGGAAG
735 E K P A S V W E Y Y I M S Q V E F R L R K S T I L K S I I N A S A
2201 TAGAAAACCTGCAAGTGTATGGGAATATTACATAATGAGCCAGTGGAGTTTGGTTGAGGAAGAGCACAATATAAGTCAATTAACACCTGATGC
768 L H L F L D E S Y L V L N Y A S Q G T V L D L I N L Q R E A K I K
2301 TTTACACTTGTCTGATGAGAGCTACCTTGTTTAACTACGCGAGTCAAGGTACGGTCTTGGATTAAATTAATTTACACGGGAAAGGCAATTGAC
801 G N G I M D E Y L C M F I T V E L M K V L E K I H E V G I I H G D L
2401 GGCAACGGAATAATGGATGAATTTTGTGATGTTTATCACTGTGCGACTAATGAAAGTACTTGAAAAGATACATGAAGTGGGAATAATACATGGCGATT
835 K P D N C M I R L E K P G E P L G A H Y M R N G E D G W E N K G I
2501 TGAAACAGATAACTGCATGATCAGATTAGAAAACAGGCGAACCCCTGGGTGCTCATTATATGCGCAATGGAGAGGATGGCTGGGAAACAGGGAAT
868 Y L I D F G R S F D M T L L P P G T K F K S N W K A D Q Q D C W E
2601 CTATTTAATAGATTTCGGTAGATCATTGATATGACACTGTACCACCTGGAACGAAATCAATCAATTTGGAAGGCTGATCAGCAAGATTGCTGGGA
901 M R A G K P W S Y E A D Y Y G L L A G V I H S M L F G K F I E T I Q L
2701 ATGCGTGGCGGCAAGCATGGAGTTATGAAGCGGATTTATGGATTAGCAGGCGTCATACATTGATGCTTTTGGAAAATTCATAGAAAACATCCAGC
935 Q N G R C K L K N P F K R Y K K E I W G V I F D L L N S Q I D
2801 TGCAGAAATGACGATGCAAAATGAAGAACCCATTCAAAAGGTTATGGAAGAAAGAAATATGGGCGTTATATTGATTACTGTTAAATAGCGGTCAAGC
968 S N Q A L P M T E K I V E I R N L I E S H L E Q H A E N H L R N V
2901 TTCCAATCAAGCTTTGCCGATGACTGAAAAGATTGTTGAAATAGGAATTTGATAGAAAGCCATCTGGAACAGCAGCAGAAAATCACTTAAGAAACGTA
1001 I L S I E E L S H F T Q Y K G G K P S R R F *** 1021
3001 ATTCTAAGCATTGAAGAGGAGTTATCATTATTTCAATATAAGGGAAACCTCAAGGAGATTTAGTGAATAAATATGCTTCATCTCCGCGATAGGTGA

```

FIG. 2. DNA sequence of the *BUB1* gene. The amino acid sequence predicted by the *BUB1* open reading frame is indicated in single-letter code. Nucleotide position 0 is the A in the predicted initiation codon. Not shown is an additional 760 bp of 5' sequence determined. There are no reasonable initiation codon candidates in this 5' region, which also includes numerous termination codons in all three reading frames.

unusually large number of amino acids between regions VI and VII. Despite a detectable similarity to serine-threonine protein kinases, none in the current GenPept database (release 79.0) is particularly closely related. The most similar relative to Bub1 is PknA, a putative protein kinase from an *Anabaena* isolate (38), which is 27% identical over 209 amino acids. The amino-terminal 700-amino-acid portion of Bub1 is not significantly related to other gene products in the database but was found to be related to the amino terminus of the product of the recently sequenced *MAD3* mitotic checkpoint gene (11) (see Discussion).

An invariant lysine corresponding to residue 733 of Bub1 has been shown to be essential for catalytic activity in many protein kinases. By site-directed mutagenesis, we made a conservative mutation of this lysine to arginine, designated

bub1K733R. We constructed two *LEU2*-containing plasmids, pTR168 (*BUB1*) and pTR170 (*bub1K733R*), that differed only at the site of this mutation (see Materials and Methods). Both were transformed into the MAY3336 diploid (*bub1Δ::URA3/BUB1*), and the resulting strains were sporulated. For pTR168, all *Ura*⁺ *Leu*⁺ spores exhibited wild-type growth rates and benomyl resistance, indicating that this construct could complement the *bub1Δ::URA3* phenotypes. In contrast, pTR170-containing *Ura*⁺ spores were still slowly growing and benomyl supersensitive, indicating that the *bub1K733R* mutant could not complement. However, *bub1K733R* was able to partially suppress the benomyl-supersensitive phenotype of the *bub1-1* putative missense allele (Fig. 5).

Genetic evidence suggests that Bub1 and Bub3 interact. The similarity of the *bub1Δ* and *bub3Δ* phenotypes and the sup-

FIG. 3. Dissected tetrads from a *bub1Δ::URA3* heterozygote. Six tetrads from strain MAY3336 (*bub1Δ::URA3/BUB1⁺*) were dissected in vertical rows onto rich medium. Each tetrad contained two *Ura⁻* (*BUB1⁺*) spores that grew into large colonies and two *Ura⁺* (*bub1Δ::URA3*) spores that were either inviable or produced slowly growing colonies. The colony color differences are due to the segregating *ade2* mutation.

pression of *bub1-1* by extra *BUB3* suggested that the products of these genes might operate in the same functional pathway. For this reason, we examined the phenotype of *bub1Δ::HIS3 bub3Δ::LEU2* double mutants. Haploid double mutants were viable but slowly growing and supersensitive to benomyl, and their phenotype was no more severe than that of the single mutants. These data contribute to the conclusion that Bub1 and Bub3 are both integral components of the same nonessential system.

High-copy-number vectors bearing either *BUB1* or *BUB3* under control of the galactose-inducible *GAL1* and *GAL10* promoters, respectively, were constructed and transformed into wild-type yeast cells. Both resulting strains grew well on galactose, although strains overexpressing *BUB1* exhibited mild benomyl sensitivity (Fig. 5). However, overexpression of both genes in the same strain resulted in a severe growth defect. These strains did not accumulate in a uniform phase of the cell cycle, and the explanation for their growth defect was not clear. Interestingly, strains overexpressing *BUB3* plus the kinase-inactive *bub1K733R* mutant exhibited a phenotype similar to but slightly stronger than that of strains overexpressing the wild-type proteins, suggesting that overphosphorylation due to the putative kinase activity of Bub1 did not cause the growth defect.

Bub1 exhibits protein kinase activity in vitro. A common feature of many protein kinases is the ability to autophosphorylate. As a means of testing whether Bub1 is an authentic protein kinase, we examined the ability of Bub1 to autophos-

PKC	GKGSFGKVMLADRKGTTEE--LYAIKILKKDVIQDDVECTMVE	
MLCK	GRGAFSIVYLGENTQT--KQRYAIKINKSELGK-DYEKNLME	
SAK	GSFSFGRVMSLQHGDNFQKCYAIKILNKEKVV-----KMKQVE	
mos	GAGGFGSVYKATYRG-VP---VAIKQVNCKTKNRLASRRSFWE	
Pkna	SGGGFGETFLAEDSQMPSNRRCVVKQLRP IHNPP--QIYQL-VQE	
Bub1p	712 GEGGYATVYLAESSQGH--LR-ALKVEKPASVW--EYYIMSQVE	750
	I	II III
PKC	GIIYRDLKLDNVMLDSEG-----HIKIADFG	
MLCK	NIVHRDLKPEILLKSKENHL-----EVAIADFG	
SAK	SLLYRDLKPEILLDLHLG-----YLVKTDFFG	
mos	SIVHLDLKPEILLISEQD-----VCKISDFG	
Pkna	RIIHRDLKPDNIILRHRDG-----KPVLDIFG	
Bub1p	828 GIIHGDLPKPDNCMIRLEKPGEPGLGAHYMYNGEDGWENKGIYLDIFG	
873		
	VI	VII
PKC	PDYIAPEIIAYQPYGKSVDDWAYG	
MLCK	PSYVAPEVLNATGYDKEVDMWSIG	
SAK	PQYLAPPEMILNRSYASQWTTGGLG	
mos	YTHRAPPELLKGEVTPKADIYSFA	
Pkna	PGYMPSEQAAGRVP YSSDLYSLG	
Bub1p	894 DQDQCWEMRAGKPPWSYEDDYGLA	917
	VIII	IX

FIG. 4. Limited comparison of primary structures of Bub1 and known protein kinases. Regions of the putative catalytic domain sequence of Bub1 are aligned with corresponding regions from known serine-threonine protein kinases. Boldface residues are those most conserved in serine-threonine protein kinases (10). PKC, protein kinase C; MLCK, myosin light-chain kinase; SAK, spermatozoan-associated kinase; mos, human *c-mos* product; Pkna, putative protein kinase from *Anabaena* sp.

phorylate in vitro. A fusion protein including the predicted amino acids 141 to 609 of *BUB1* (amino terminal to the putative kinase domain) was expressed in *E. coli*, purified, and injected into rabbits. Affinity-purified polyclonal antisera from these rabbits specifically recognized a group of proteins with apparent molecular masses ranging from approximately 125 to 135 kDa and several smaller proteins (Fig. 6). The predicted molecular mass of Bub1 is 118 kDa. (In addition, a band of approximately 65 kDa was nonspecifically recognized by this affinity-pure preparation.) Note that these protein bands were evident only in extracts from cells overexpressing *BUB1* (either along with overexpressed *BUB3* as shown or in cells with wild-type Bub3 levels [not shown]), not from wild-type yeast cells, and were not recognized by preimmune serum (Fig. 6). These specific bands were also completely absent from cells expressing a *BUB1* gene truncated by removing the two 3' *Pst*I fragments (data not shown). Taken together, these observations indicate that these antibodies specifically recognize cellular forms of Bub1. The affinity-pure antibodies were used to immunoprecipitate Bub1 from extracts made from overproducing cells. These precipitates were Western blotted and probed with the same antibodies. Only bands corresponding to 125 and 130 kDa were observed, although the dense band caused by the precipitating antibody heavy chains might have obscured forms near 50 kDa (Fig. 7 and 8 and data not shown). It is likely that the other molecular mass forms of Bub1 visible in the Fig. 6 Western blot are proteolytic fragments or other short-lived forms.

These antibodies were used to immunoprecipitate overproduced Bub1 from the soluble portion of whole cell lysates. One-third of the immune complex sample was incubated in a buffer containing $MgCl_2$ and $[\gamma\text{-}^{32}P]\text{ATP}$ and then subjected to SDS-PAGE and autoradiography (Fig. 7). The remaining two-thirds were subjected to SDS-PAGE, then blotted to nitrocellulose, and probed with antibodies to Bub1. The autophos-

TABLE 2. Cell morphologies on benomyl-containing medium

Strain	Genotype	% of cells with indicated no. of cell bodies ^a			
		2	3	4	>4
MAY589	<i>BUB⁺</i>	89	9	2	0
MAY3594	<i>bub1Δ::URA3</i>	47	37	4	12
MAY3598	<i>bub3Δ::LEU2</i>	54	41	4	1

^a Values are percentages of total cells exhibiting the indicated number of cell bodies after release from α -factor arrest followed by 6 h on benomyl (70 $\mu\text{g/ml}$)-containing solid medium. Immediately after release from α -factor, 88 to 94% of the cells had unbudded morphologies (one cell body).

FIG. 5. Growth and rescue of *bub* mutants and strains overproducing Bub proteins. Strains were derepressed on synthetic media with raffinose and then plated to glucose or galactose medium containing the concentration of benomyl (in micrograms per milliliter) indicated at the bottom (see Materials and Methods for details of plasmid construction and growth conditions). For each horizontal row of cells, the chromosomal genotype is indicated followed by the relevant plasmid-borne genes. pGAL indicates that the following gene is expressed from a galactose-inducible promoter. Note that both galactose promoter-driven *BUB1* and *BUB3* express sufficient gene product on glucose medium to cause phenotypic consequences. All plasmids have the *URA3* marker. pBUB3 is a *CEN* vector, but all other plasmids are multicopy 2- μ m vectors derived from YEp352. Strains: *bub1-1* YEp352, MAY3419; *bub1-1* pBUB3, MAY1800; *bub1-1* pGAL-BUB1, MAY3421; *bub1Δ* YEp352, MAY3337; *bub1Δ* pBUB3, MAY3420; *bub1Δ* pGAL-BUB3, MAY3339; *bub3Δ* YEp352, MAY3343; *bub3Δ* pGAL-BUB1, MAY3316; *bub3Δ* pGAL-BUB3, MAY3340; *BUB+* YEp352, MAY3317; *BUB+* pGAL-BUB1, MAY3335; *BUB+* pGAL-BUB3, MAY3341; *BUB+* pGAL-BUB1 pGAL-BUB3, MAY3188; *BUB+* pGAL-bub1K733R pGAL-BUB3, MAY3275; *bub1-1* pGAL-bub1K733R, MAY3422; *bub1Δ* pGAL-bub1K733R, MAY3423.

phorylation experiment revealed a major phosphate-labeled band of approximately 130 kDa. In the parallel immunological detection experiment, anti-Bub1 antibodies detect a doublet of proteins corresponding to molecular masses of 125 and 130 kDa. No bands of this size were observed in either experiment when preimmune serum was substituted for immune serum in the immunoprecipitation. Furthermore, none of these bands were observed unless *BUB1* was overexpressed in the cells from which the extracts were made. Therefore, we conclude that 130-kDa phosphoprotein and the immunologically detected doublet represent forms of the authentic *BUB1* gene product.

Since it was possible that the observed phosphorylation of Bub1 was catalyzed by a contaminating protein kinase, the experiment was also performed with the *bub1K733R* mutant described above. When the mutant allele was overexpressed, it produced a protein of the size of Bub1 that was visible by Western analysis. No significant incorporation of 32 P was observed for this protein (Fig. 7A, lane 4). Note that in some experiments with this mutant, a very faint band was observed at the molecular mass of Bub1 (data not shown). This weak signal may be due to the presence of a contaminating protein kinase in the immunoprecipitate or possible low residual activity of the mutant gene product. Although the lysine-to-arginine substitution is highly conservative, it appears to reduce the stability of the protein, and consequently a smaller amount mutant protein was immunoprecipitated from an equivalent amount of cell lysate. To compensate for this in the experiment, a portion of the wild-type Bub1 complexes was diluted fivefold and analyzed next to the *bub1K733R* mutant. Even in the diluted wild-type lane (Fig. 7A, lane 3), 32 P incorporation was much higher than for the *bub1K733R* mutant. These data indicate that Bub1 exhibits protein kinase activity in vitro.

Bub3 physically interacts with and is a substrate for the Bub1 protein kinase in vitro. Several smaller proteins, ranging from approximately 30 to 90 kDa, were also phosphate labeled in the Bub1 immunoprecipitates (Fig. 7A and 8A). These could represent other coimmunoprecipitating substrates, degraded forms of phosphorylated Bub1, or even phosphorylated antibody chains. Since Bub3 has a predicted molecular mass of 39 kDa, we tested the hypothesis that the 40-kDa protein prominently labeled in the precipitates represented the *BUB3* gene product. Although the 40-kDa protein band could sometimes be seen when *BUB3* was expressed from its chromosomal promoter, the band increased in intensity when *BUB3* was overexpressed along with *BUB1* (Fig. 8A). When the autophosphorylation assay was performed with extracts from *bub3* deletion mutants overexpressing *BUB1*, the 40-kDa band was absent. Furthermore, a protein of the same molecular mass was recognized by Bub3 antibodies in Bub1 immunoprecipitates (Fig. 7B and 8B). We conclude from these observations that Bub1 and Bub3 coimmunoprecipitate and that the *BUB3* gene product is an in vitro substrate for Bub1.

Overexpression of *BUB3* along with *BUB1* resulted in a consistently higher level of labeling of all proteins phosphorylated in the experiment compared with overproduction of Bub1 alone (Fig. 8A, lane 1 versus lane 2). Although overproduction of Bub3 tended to boost the amount of Bub1 immunoprecipitated in some trials (data not shown), in this experiment a comparable amount of Bub1 was loaded in lanes 1 to 3, as indicated by Western analysis (Fig. 8B). However, autophosphorylation of Bub1 did not depend on the presence of Bub3 in the cell extract; Bub1 phosphorylation was still observed when it was immunoprecipitated from a *bub3* deletion mutant (Fig. 8A, lane 3). These data suggest that Bub3 has an activating effect on the Bub1 kinase but is not essential for its

FIG. 6. Specificity of affinity-purified anti-Bub1 antiserum. Whole cell protein extracts from wild-type cells (MAY3317; lanes 1 and 3) or cells overexpressing *BUB1* and *BUB3* (MAY3188; lanes 2 and 4) were subjected to SDS-PAGE and transferred to nitrocellulose. Lanes 1 and 2 were probed with affinity-purified polyclonal antibodies raised to a portion of the *BUB1* gene product (see Materials and Methods). Lanes 3 and 4 were probed with affinity-purified preimmune serum from the same rabbit. Numbers at the left indicate approximate molecular masses in kilodaltons. The predicted molecular mass of full-length, unphosphorylated Bub1 is 118 kDa.

enzymatic activity. Note, however, that cells overproducing Bub1 and Bub3 together are inhibited for growth relative to those overproducing Bub1 alone. Cells devoid of Bub3 are also slowly growing but possibly for a different reason. Therefore,

FIG. 7. Autophosphorylation assay. (A) Autoradiograph of immune complexes incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (see Materials and Methods). (B) Western blot of the same immune complexes, probed with an anti-Bub1 ($\alpha\text{-Bub1p}$) or anti-Bub3 ($\alpha\text{-Bub3p}$) antibody to assess recovery of these proteins. For both panels, immune complexes in lane 1 were formed with affinity-purified preimmune serum; all other immune complexes were from affinity-purified anti-Bub1 serum. Protein extracts were from wild-type strains with pGAL-*BUB1* pGAL-*BUB3* (MAY3188; lanes 1 and 2), pGAL-*bub1K733R* pGAL-*BUB3* (MAY3275; lane 4), or vector only (MAY3317; lane 5). Lane 3 is a fivefold dilution of lane 2. Approximate molecular mass standards for panel A are indicated (in kilodaltons) at the left. Bands in panel B are aligned with the corresponding positions in panel A.

FIG. 8. Interaction of Bub3 with, and phosphorylation by, Bub1. (A) Autoradiograph of phosphorylated proteins from anti-Bub1 immune complexes; (B) Western analysis of the same immune complexes, to judge recovery of Bub1 ($\alpha\text{-Bub1p}$) and Bub3 ($\alpha\text{-Bub3p}$). In panels A and B, protein extracts were made from *BUB*⁺ pGAL-*BUB1* pGAL-*BUB3* (lane 1), *BUB*⁺ pGAL-*BUB1* (lane 2), and *bub3Δ* pGAL-*BUB1* (lane 3). (C) Coimmunoprecipitation of Bub3 with a fusion protein from the amino-terminal portion of Bub1. Both lanes represent immunoprecipitations with anti-Bub1 antibodies from *BUB*⁺ pGAL-*BUB3* cells (MAY3341). In lane 2, approximately 1 μg of a 64-kDa Bub1 partial fusion protein was added to the yeast protein extracts. Immune complexes were subjected to SDS-PAGE, blotted to nitrocellulose, and probed with the anti-Bub3 antibody.

the differences in Bub1 kinase activity observed in extracts from cells with various amounts of Bub3 may be an indirect consequence of physiological differences.

The region of Bub1 between amino acids 141 and 609 is sufficient for interaction with Bub3. It seemed possible that the amino-terminal portion of Bub1 contains a domain required for binding to Bub3. We tested the ability of a fusion protein

FIG. 9. Immunofluorescent staining of Bub1 in wild-type and *BUB*-overexpressing cells. Cells in panel A are labeled with affinity-pure anti-Bub1 antibody ($\alpha\text{-Bub1p}$) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody; cells in panel B are labeled with the fluorescent DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI). Both show the same field of pGAL-*BUB1* pGAL-*BUB3* cells (strain MAY3188) 12 h after induction with galactose (see Materials and Methods).

purified from *E. coli* including amino acids 141 through 609 of Bub1 to interact with Bub3. Note that this protein is the same as the protein used to generate the polyclonal anti-Bub1 antibodies used in this study. When immunoprecipitations were performed with these antibodies on extracts from cells overexpressing *BUB3* alone, Bub3 was not observed in the precipitate. However, when the fusion protein was added to the same protein extract before addition of antibodies, Bub3 was precipitated (Fig. 8C). These data indicate that a protein-protein binding domain sufficient for interaction with Bub3 is located between amino acids 141 and 609 of Bub1.

Overproduction of Bub1 results in its accumulation in the nucleus. The benomyl sensitivity caused by the overproduction of Bub1 and other evidence indicating a genetic interaction between Bub1, Bub3, and tubulin (9) (see Discussion) suggested that Bub1 might physically associate with microtubules. To determine the cellular localization of Bub1, *in situ* immunofluorescence was performed with affinity-pure antibodies against Bub1. This treatment revealed heavy nuclear staining in cells overexpressing *BUB1* and *BUB3*, but microtubule-specific staining was not detected (Fig. 9). This pattern was also observed in cells overexpressing *bub1K733R* and *BUB3* together and in *bub3Δ* and *BUB3* cells overexpressing *BUB1* alone (data not shown). No staining was detected in cells producing wild-type levels of Bub1.

DISCUSSION

The *BUB1* and *BUB3* genes were identified in a search for genes required for normal control of mitosis. Both proteins have been shown to be required for cell cycle arrest in response to spindle disruption (14). Several lines of evidence suggested that Bub1 and Bub3 act as part of a single pathway to maintain normal cell cycle timing. First, *BUB3* was originally cloned as an extra-copy suppressor of *bub1-1*. Second, the phenotypes of the *bub1Δ bub3Δ* double-deletion mutant were similar or equivalent to those of either single mutant. These mutants were viable but extremely slowly growing and sensitive to benomyl. Third, overexpression of the two genes together led to a sharp reduction in growth rate, although overproduction of the single gene products did not inhibit growth under permissive conditions. Fourth, these genes are each capable of allele-specific suppression of the *tub1-729* mutation when present in extra copy (9). Despite this genetic link, there is still no direct evidence for interaction of either Bub1 or Bub3 with tubulin. Finally, the two proteins coimmunoprecipitated, and the presence of Bub3 seemed to increase the *in vitro* activity of the Bub1 kinase.

The phenotypes of *bub1* and *mad3* mutants are similar (14, 20). Both exhibit premature cell cycle progression when the function of the mitotic spindle is compromised. The recently determined sequence of *MAD3* revealed that the predicted amino terminus of its product is closely related to the amino terminus of Bub1 (11). The first 360 amino acids of Bub1 are 35% identical to the corresponding region of Mad3, and this region includes a 147-amino-acid sequence that is 44% identical. This region overlaps with the region of Bub1 that we found is sufficient to interact with Bub3 *in vitro* (amino acids 141 through 609), suggesting the possibility that Mad3 also binds Bub3. Protein kinase consensus sequences were not found in the predicted *MAD3* gene product.

Bub1 and Bub3 activities are required to prevent premature exit from M phase when mitotic spindle function is compromised by microtubule depolymerization (14). It is possible that under these conditions, the Bub1 kinase is activated in some sort of signal transduction capacity. We have been unable to

detect any difference in Bub1 kinase activity between extracts obtained from nocodazole-treated and untreated cells (unpublished observations). However, it is possible that the overproduction of Bub1 necessary for its detection in this experiment overshadowed any normal *in vivo* regulatory effects. We can suggest two potential mechanisms of catalytic regulation of the Bub1 kinase. First, Bub1 is a member of a small subset of protein kinases with a tyrosine residue in the ATP binding site corresponding to position 716 in Bub1. Most of the other members of this group are closely related to p34^{cdc2}, the catalytic subunit of the MPF kinase (10). In some eukaryotes, MPF kinase activity is negatively regulated by phosphorylation of this tyrosine (3, 22–24). Although p34^{cdc2} is not a close relative of Bub1, it is possible that Bub1 is also regulated by phosphorylation of tyrosine 716. Second, it has been shown that catalytic regulation of some protein kinases is achieved through interaction of an autoinhibitory domain of the protein with the catalytic site (32). Kinase activity is stimulated by association with an allosteric activator. Bub3 may be such an activator of Bub1 or may direct the activation of Bub1 by an unidentified protein. Since the *BUB1* gene product is somewhat divergent from other known protein kinases, however, an alternative hypothesis is that Bub3 is a substrate-binding subunit within the Bub1-Bub3 kinase complex. In light of the fact that Bub1 produced in *bub3Δ* mutants is capable of autophosphorylation, this hypothesis seems highly unlikely. Bub3 may, however, direct association of Bub1 to its physiological substrate.

Elimination of MPF activity has been suggested to be required for exit from mitosis (8, 35). Since MPF activity has been shown to be improperly regulated in the *bub* mutants (14), the various MPF subunits may reasonably be viewed as candidate substrates and/or downstream effectors for Bub1 and Bub3. Many of the cyclin subunits of MPF are known to be phosphorylated *in vivo*. Although phosphorylation of *Xenopus* B-type cyclins has been shown to be nonessential for normal cell cycle function (15), a role for their phosphorylation in checkpoint control has not been ruled out.

In *S. pombe*, negative regulation of MPF activity is achieved in part through the phosphorylation of p34^{cdc2} on tyrosine 15 and threonine 14 (3, 22–24). Although the equivalent residues are phosphorylated with similar cell cycle kinetics on the *S. cerevisiae* homolog Cdc28 (2, 34), these phosphorylations are dispensable for normal arrest in G₁, S, or M phase. Indeed, mutations causing the change of the threonine and tyrosine to nonphosphorylatable residues has no influence on cell cycle timing or cell viability. In light of the deleterious nature of *bub1* or *bub3* mutations and their negative influence on MPF activity (14), it is unlikely that this Bub pathway influences cycle timing by affecting the phosphorylation state of these residues. In *S. pombe*, as well as other eukaryotes, the p40^{MO15} kinase is believed to catalyze phosphorylation of a third site on p34^{cdc2} (equivalent to *S. cerevisiae* position Thr-160), an event required for its activation (6, 26, 33). Since a p40^{MO15} functional homolog has not been identified in yeast isolates, Bub1 may be the primary *S. cerevisiae* Thr-160 kinase. Note, however, that sequence similarity between these kinases is limited. If not equivalent to Bub1, the yeast functional homolog of p40^{MO15} may be a candidate target for regulation by the Bub pathway. Finally, since it is clear that Bub3 is phosphorylated by Bub1 *in vitro*, Bub3 itself may be the only relevant substrate of Bub1.

ACKNOWLEDGMENTS

We thank Suzanne Guénette and Frank Solomon for the anti-Bub3 antibodies and communication of unpublished results and Kevin

Hardwick and Andrew Murray for communicating the Mad3 sequence. We also thank Laura Totis for technical assistance, David Levin for suggestions on the kinase assays, and John Geiser, Kevin Hardwick, David Levin, Jim Maller, Bill Saunders, and Laura Scott for comments on the manuscript.

This work was supported by NIH grant GM49363 awarded to M.A.H.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Amon, A., U. Surana, I. Muroff, and K. Nasmyth. 1992. Regulation of p34^{CDC28} tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature (London)* **355**:368–371.
- Atherton-Fessler, S., L. Parker, R. L. Geahlen, and H. Piwnica-Worms. 1993. Mechanisms of p34^{cdc2} regulation. *Mol. Cell. Biol.* **13**:1675–1685.
- Celenza, J. L., and M. Carlson. 1986. A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233**:1175–1180.
- Connelly, C., and P. Hieter. Unpublished data.
- Dustin, P. 1984. Microtubules. Springer-Verlag, Berlin.
- Fesquet, D., J. Labbé, J. Derancourt, J. Capony, S. Galas, F. Girard, T. Lorca, J. Shuttleworth, M. Dorée, and J. Cavadore. 1993. The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J.* **12**:3111–3121.
- Frankhauser, C., J. Marks, A. Reymond, and V. Simanis. 1993. The *S. pombe cdc16* gene is required both for maintenance of p34^{cdc2} kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *EMBO J.* **12**:2697–2704.
- Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed. 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase prevents exit from mitosis. *Cell* **65**:163–174.
- Guénette, S., M. Magendantz, and F. Solomon (Massachusetts Institute of Technology). 1994. Personal communication.
- Hanks, S. K., and A. M. Quinn. 1991. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* **200**:38–62.
- Hardwick, K., and A. W. Murray. (University of California, San Francisco). 1994. Personal communication.
- Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**:629–634.
- Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff. 1986. *Yeast/E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**:163–167.
- Hoyt, M. A., and B. T. Roberts. Unpublished data.
- Hoyt, M. A., L. Totis, and B. T. Roberts. 1991. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**:507–517.
- Izumi, T., and J. L. Maller. 1991. Phosphorylation of *Xenopus* cyclins B1 and B2 is not required for cell cycle transitions. *Mol. Cell. Biol.* **11**:3860–3867.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
- Langan, T. A., J. Gautier, M. Lohka, R. Hollingsworth, S. Moreno, P. Nurse, J. Maller, and R. A. Sclafani. 1989. Mammalian growth-associated H1 histone kinase: a homolog of *cdc2*⁺/*CDC28* protein kinases controlling mitotic entry in yeast and frog cells. *Mol. Cell. Biol.* **9**:3860–3868.
- Li, R., and A. W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell* **66**:519–531.
- Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* **256**:11955–11957.
- Parker, L. L., S. Atherton-Fessler, M. S. Lee, S. Ogg, J. L. Falk, K. I. Swenson, and H. Piwnica-Worms. 1991. Cyclin promotes the tyrosine phosphorylation of p34^{cdc2} in a *wee1*⁺ dependent manner. *EMBO J.* **10**:1255–1263.
- Parker, L. L., S. Atherton-Fessler, and H. Piwnica-Worms. 1992. p107^{wee1} is a dual-specificity kinase that phosphorylates p34^{cdc2} on tyrosine 15. *Proc. Natl. Acad. Sci. USA* **89**:2917–2921.
- Parker, L. L., and H. Piwnica-Worms. 1992. Inactivation of the p34^{cdc2}-cyclin B complex by the human WEE1 tyrosine kinase. *Science* **257**:1955–1957.
- Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
- Poon, R., K. Yamashita, J. Adamczewski, T. Hunt, and J. Shuttleworth. 1993. The *cdc2*-related protein p40^{MO15} is the catalytic subunit of a protein kinase that can activate p33^{cdk2} and p34^{cdc2}. *EMBO J.* **12**:3123–3132.
- Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* **194**:565–602.
- Riles, L., and M. V. Olson. Personal communication.
- Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101**:202–211.
- Schatz, P. J., F. Solomon, and D. Botstein. 1986. Genetically essential and nonessential α -tubulin genes specify functionally interchangeable proteins. *Mol. Cell. Biol.* **6**:3722–3733.
- Sherman, F., G. R. Fink, and J. B. Hicks. 1983. Methods in yeast genetics. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
- Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
- Soderling, T. R. 1990. Protein kinases: regulation by autoinhibitory domain. *J. Biol. Chem.* **265**:1823–1826.
- Solomon, M. J., J. W. Harper, and J. Shuttleworth. 1993. CAK, the p34^{cdc2} activating kinase, contains a protein identical or closely related to p40^{MO15}. *EMBO J.* **12**:3133–3142.
- Sorger, P. K., and A. W. Murray. 1992. S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34^{CDC28}. *Nature (London)* **355**:365–368.
- Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth. 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* **12**:1969–1978.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
- Weinert, T. A., and L. H. Hartwell. 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241**:317–322.
- Zhang, C. C. 1993. A gene encoding a protein related to eukaryotic protein kinases from the filamentous heterocystous cyanobacterium *Anabaena* PCC 7120. *Proc. Natl. Acad. Sci. USA* **90**:11840–11844.