Hypomorphic \textit{bimA}^{APC3} Alleles Cause Errors in Chromosome Metabolism That Activate the DNA Damage Checkpoint Blocking Cytokinesis in \textit{Aspergillus nidulans}

Tom D. Wolkow,* Peter M. Mirabito,¹ Srinivas Venkatram¹ and John E. Hamer*

*Department of Biology, Purdue University, West Lafayette, Indiana 47907-1392 and ¹Molecular and Cellular Biology Section, School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506-0225

Manuscript received July 2, 1999
Accepted for publication August 25, 1999

ABSTRACT

The \textit{Aspergillus nidulans} \textit{sep} \textit{I} \textsuperscript{+} gene has been implicated in the coordination of septation with nuclear division and cell growth. We find that the temperature-sensitive (ts) \textit{sep} \textit{I} mutation represents a novel allele of \textit{bimA}^{APC3}, which encodes a conserved component of the anaphase-promoting complex/cyclosome (APC/ C). We have characterized the septation, nuclear division, cell-cycle checkpoint defects, and DNA sequence alterations of \textit{sep} \textit{I} (renamed \textit{bimA10}) and two other ts lethal \textit{bimA}^{APC3} alleles, \textit{bimA1} and \textit{bimA9}. Our observations that \textit{bimA9} and \textit{bimA10} strains have morphologically abnormal nuclei, chromosome segregation defects, synthetic phenotypes with mutations in the DNA damage checkpoint genes uvs\textit{B} \textit{dic} \textit{Y}/rad3 \textit{ts} or uvs\textit{D} \textit{ts}, and enhanced sensitivity to hydroxyurea strongly suggest that these strains accumulate errors in DNA metabolism. We found that the aspartate phenotype of \textit{bimA9} and \textit{bimA10} strains was substantially relieved by mutations in uvs\textit{B} \textit{dic} \textit{Y}/rad3 \textit{ts} or uvs\textit{D} \textit{ts}, suggesting that the presence of a functional DNA damage checkpoint inhibits septation in these \textit{bimA}^{APC3} strains. Our results demonstrate that mutations in \textit{bimA}^{APC3} lead to errors in DNA metabolism that indirectly block septation.

CYTOKINESIS is temporally coordinated with the nuclear division cycle to help ensure the proper segregation of genetic material and cytoplasm to daughter cells. The strict temporal coupling between mitosis and cytokinesis complicates genetic and biochemical studies of this coordination (reviewed by Satter white and Pollard 1992) because mitotic regulatory molecules likely control both processes by distinct mechanisms. For instance, studies in both animal and fungal cells suggest that cyclin-dependent kinase activity coordinates both events by suppressing cytokinesis until exit from mitosis has begun (Satter white et al. 1992; Wheatley et al. 1997; reviewed by Fishkind and Wang 1995; Gould and Simanis 1997).

Septum formation in \textit{Aspergillus nidulans} affords a unique opportunity to study cytokinesis as an event temporally separated from, although dependent on, the nuclear division cycle. Conidia of \textit{A. nidulans} have a single nucleus arrested in G1 of the cell cycle (Bergen and Morris 1983). Following germination, nuclear division and germ tube extension occur. Three rounds of nuclear division (producing eight nuclei) take place before the onset of septation (Fiddy and Trinci 1976; Harris et al. 1994). Experiments with various mitotic mutants have demonstrated that this pattern of growth, nuclear division, and septation are dependent on controls that ensure that germlings acquire a minimum threshold cell size and undergo at least one nuclear division before cytokinesis. Thus, small germlings having undergone only one or two rounds of nuclear division are aseptate (Wolkow et al. 1996). Septation in \textit{A. nidulans} is also dependent upon a contractile actin ring. Both formation and contraction of the actin ring require intact microtubules, while positioning of the ring is influenced by the distribution of nuclei (Wolkow et al. 1996; Momany and Hamer 1997).

To identify genes involved in cytokinesis, temperature-sensitive (ts) mutants, which do not septate after germ tube extension and multiple rounds of nuclear division, were identified (Harris et al. 1994). \textit{sep} (septation defective) mutants are operationally grouped into distinct classes termed “early” or “late.” Late mutants (\textit{sep} \textit{A}, \textit{D}, G, and \textit{H}) undergo continuous nuclear division and apical growth at the restrictive temperature but fail to septate. Early mutants (\textit{sep} \textit{B}, \textit{E}, I, and \textit{J}) undergo only approximately three nuclear divisions in the presence of an extending germ tube before arresting growth as aseptate cells. Unlike late mutants, early \textit{sep} mutants do not septate after return to permissive temperature, suggesting these mutants are unable to initiate early events of the septation process.

The early \textit{sep} \textit{B} \textit{ts} gene encodes an essential protein required for efficient chromosome segregation (Harris and Hamer 1995). \textit{sep} \textit{B} \textit{ts} mutants accumulate defects in chromosome metabolism that eventually prevent the initiation of septation and lead to growth arrest. It has
recently been shown that the aspate phenotype of sepB3 and sepI1 germlings requires components of the DNA damage checkpoint (Harris and Kraus 1998). In addition, DNA-damaging agents and genetically elevated levels of Tyr-15-phosphorylated p34(SIMX) also inhibit septation (Harris and Kraus 1998; X. Ye, T. Wol-
kow, A. Tang, R. Fincher, S. L. McQuire, J. E. Hamer and S. A. Osmani, unpublished results). These findings show that a checkpoint exists in A. nidulans that delays or even blocks cytokinesis in response to accumulated DNA damage. The finding that irradiated p53-/-m and p21-/- human cells progress through mitosis but block cytokinesis (Bunz et al. 1998) suggests that this checkpoint may be a conserved feature of eukaryotic cells and occurs in a p53-independent fashion.

To gain further insight into the mechanisms linking nuclear division and cytokinesis, we characterized the early sepI1 mutant and cloned sepI1. Here we show that sepI1 is an allele of bimA<sup>APC3</sup>, which encodes a component of the anaphase-promoting complex/cyclosome (APC/C; Her shko et al. 1994; Petit et al. 1996). The APC/C is a ubiquitin ligase required for timely initiation of S phase (Amon et al. 1994; Brandeis and Hunt 1996; Irniger and Nasmuth 1997; Ye et al. 1997b) as well as anaphase progression and mitotic exit (reviewed by King et al. 1996; Townsley and Ruderman 1998). It has also been implicated in cell-cycle checkpoints, including the spindle assembly (He et al. 1997; Li et al. 1997; Fang et al. 1998; Kallio et al. 1998; reviewed by Rudner and Murray 1996) and DNA damage checkpoints (Cohen-Fix and Kosland 1997) as well as the S-phase (Ye et al. 1996) and G2 checkpoints (Lies et al. 1998) of A. nidulans (reviewed by Osmani and Ye 1997).

To investigate the mechanism by which bimA<sup>APC3</sup> mutations affect septation, the three existing bimA alleles (bimA9 and bimA10 (sepI1)) were characterized. Sequence alterations of the alleles were found to occur in different regions of bimA<sup>APC3</sup>. Each allele produced phenotypes consistent with APC/C defects, such as aberrant mitotic progression and chromosome segregation, as well as failure to promote G<sub>2</sub> arrest in the absence of normal NIMA function. Unlike bimA1, which has been shown to cause a metaphase arrest, both bimA9 and bimA10 caused early sept arrest. The phenotypic and genetic analyses presented here demonstrate that the accumulation of chromosome metabolism errors in bimA9 and bimA10 germlings activates a DNA damage checkpoint that blocks septation.

**MATERIALS AND METHODS**

**Strains and growth conditions:** Strains of A. nidulans used in this study are listed in Table 1. CM media is 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5. Trace elements, vitamins, and nitrate salts are described in the appendix to Kafer (1977). For solid media, 1.8% agar was added. Genetic techniques for A. nidulans are described in Harris et al. (1994). Strains designated nimA-alc contained alcA::nimA as the only functional nimA gene. Culture conditions for propagation and analysis of these strains are as described previously (Lies et al. 1998).

To observe phenotypes, supplemented CM liquid was inoculated with 1–5 x 10<sup>5</sup> conidia/ml, poured into a petri dish containing glass coverslips (coverslip cultures), and incubated at 30<sup>°</sup> for permissive temperature and 42<sup>°</sup> or 46.5<sup>°</sup> for restrictive temperatures.

To follow nuclear division kinetics and chromosome mitotic index (%CMI) in germlings, coverslip cultures were incubated at 42<sup>°</sup>. At various intervals, coverslips were removed, fixed, and stained with Hoechst 33258 (Polysciences, Warrington, PA). To determine the percentage of germlings with septa, coverslip cultures were incubated at 42<sup>°</sup> for 10 hr. These germlings were stained with both Hoechst 33258 and Calcofluor (a gift from American Cyanamid) to observe nuclei and septa, respectively. Only germlings having at least eight nuclei in an extended germ tube were assayed for the presence of a septum. All experiments were repeated at least two times with similar results, and results from one experiment are shown.

**Sensitivities of bima<sup>APC3</sup>, bimA9, and bimA10 to hydroxyurea (HU) were examined at permissive temperature using CM agar medium containing different concentrations of the drug (4, 6, 8, 10, or 15 mm HU). Conidia were plated at a concentration of ~200 spores per plate and allowed to grow for 4 days at 30<sup>°</sup>.

**Recombinant DNA and transformation techniques:** The molecular biology techniques of Sambrook et al. (1989) were used throughout this study. DNA-mediated transformations and isolation of genomic DNA from A. nidulans were also accomplished using previously described methods (Timmerlake 1990; Dobinson et al. 1993; O'akley and Osmani 1993).

**Cloning of the sepI1 gene:** Strain AJM86 was cotransformed with pDHG25 (Arg<sup>+</sup> plasmid; kindly provided by Dr. J. Clutterbuck, University of Glasgow) and a chromosome I-specific cosmid library (Brady et al. 1991). Arg<sup>+</sup> transformants were replica plated to MN plates and incubated at 42<sup>°</sup> to select for Ts<sup>+</sup> transformants. Additional transformation experiments revealed that complementing activity resided on a 3.5-kb Sad fragment of cosmid L20D04. This Sad fragment was sequenced and found to contain the 3<sup>‘</sup> region of bima<sup>APC3</sup> (O'Donnell et al. 1991).

**Staining and microscopy:** Fixing, staining, and microscopy methods are described by Harris et al. (1994). The immunofluorescence microscopy technique used to detect microtubules has been described previously (Osmani and Ye 1993). The primary antibody used was mouse anti-tubulin DNA monoclonal (Sigma, St. Louis) at 1:200. The secondary antibody used was Texas red-goat anti-mouse IgG TRITC (Molecular Probes, Eugene, OR).

**Isolation and sequencing of bima alleles:** Genomic DNA was prepared from A28, AJM86, MLC1-19, and PM144. Vent DNA polymerase (New England Biolabs, Beverly, MA) was used for PCR amplifications. Three overlapping sets of primers were used to amplify genomic DNA. Primer names and sequences are as follows: Bim1 (5'-CCG GTA TCG CCT TGA TCC CGT TCC TGA TGA TGC CAC-3'), Bim2 (5'-GCC CTT AAG TCC TGT TCC TGA AGA TGC CAC-3'), Bim3 (5'-CCG GTA TCG CCT TCG ACA ACA CTG AGT-3'), Bim4 (5'-GCC CTT AAG CCA AAG AAC CGT GTA GAT CTC-3'), Bim5 (5'-CCG GTA TCG CCT TCG CCC TGT GTA GAT-3'), and Bim6 (5'-GCC CTT AAG GTA GAA GTA GGA TGG CTG AA-3'). Two independent clones of each allele were sequenced multiple (more than two) times on both strands.

**RNA transcript analysis:** RNA was extracted from 0.1 g of lyophilized mycelium using TRIzol reagent and the accompanying protocol (Life Technologies). RQ1 RNase-Free DNase


### TABLE 1

**A. nidulans strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A28*</td>
<td>pabaA6 bimA1 veA1</td>
</tr>
<tr>
<td>A362*</td>
<td>riboA1 anA1 adG14 proA1 pabaA1 ya2 veA1</td>
</tr>
<tr>
<td>A852*</td>
<td>pabaA1 ya2 ArgB:trpCΔB; veA1 trpC801</td>
</tr>
<tr>
<td>AJM86</td>
<td>bimA10 argB2 veA1</td>
</tr>
<tr>
<td>ASH 60</td>
<td>sepB3; veA1</td>
</tr>
<tr>
<td>ASH 201*</td>
<td>uvsB110; nicA2; chaA1 veA1</td>
</tr>
<tr>
<td>ASH 206*</td>
<td>bimA1; wa2; uvsD153; veA1</td>
</tr>
<tr>
<td>ATW41</td>
<td>pabaA1 bimA10 veA1</td>
</tr>
<tr>
<td>ATW43</td>
<td>bimA10 pabaA1; argB2; veA1/bimA1+ pabaA1; argB+ veA+</td>
</tr>
<tr>
<td>ATW53</td>
<td>bimA10 pabaA1; argB2/bimA10 pabaA1; argB+</td>
</tr>
<tr>
<td>ATW55</td>
<td>bimA9 pabaA1; wa2; uvsD153; veA1</td>
</tr>
<tr>
<td>ATW56</td>
<td>bimA1 bimA10; uvsD153; veA1</td>
</tr>
<tr>
<td>ATW57</td>
<td>bimA10; uvsB110; veA1</td>
</tr>
<tr>
<td>ATW61</td>
<td>bimA10; argB2; veA1/bimA9 pabaA1 ya2; nicA2; veA1</td>
</tr>
<tr>
<td>ATW62</td>
<td>bimA10 pabaA1; veA1/bimA1; argB2; nicA2; veA1</td>
</tr>
<tr>
<td>ATW63</td>
<td>pyrG- bimA9 pabaA1 ya2; nicA2; veA1/pyrG99 bimA1+ pabaA1+ ya2+ nicA1+; veA-</td>
</tr>
<tr>
<td>ATW64</td>
<td>riboA1 anA1 adG14 pabaA1+ ya2/roboA1+ anA+ adG+ pabaA1 ya+</td>
</tr>
<tr>
<td>ATW66</td>
<td>bimA9; uvsB110; nicA2; veA1</td>
</tr>
<tr>
<td>MLC1-19</td>
<td>bimA9 pabaA1 ya2; nicA2; veA1</td>
</tr>
<tr>
<td>PM156</td>
<td>bimA1; wa2; veA1</td>
</tr>
<tr>
<td>SFC552-28</td>
<td>bimA10; argB2; wa2; pyroA1; nicA2 [argB+ alcA::nimA1 at argB]; [pyr4+ nimAΔ]</td>
</tr>
<tr>
<td>SFC466-48</td>
<td>bimA1 pyrG99; argB2; nicA2; chaA1; chaA1 [argB+ alcA::nimA at argB]; [pyr4+ nimAΔ]</td>
</tr>
<tr>
<td>SFC466-201</td>
<td>pyrG99; argB2; nicA2; wa2 [argB+ alcA::nimA at argB] [pyr4+ nimAΔ]</td>
</tr>
<tr>
<td>SFC470-1</td>
<td>bimA9 pyrG99; argB2; nicA2 [argB+ alcA::nimA at argB] [pyr4+ nimAΔ]</td>
</tr>
</tbody>
</table>

* Obtained from Fungal Genetic Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66160-7420.
* Obtained from Dr. Steven Harris, Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205.
* Genotype entered in brackets indicates that the strain carries a plasmid that contains the argB+ gene and an alcA::nimA gene fusion at the argB locus.
* [pyr4+ nimAΔ] indicates that the nimA gene has been deleted and replaced with the pyr4 gene, as described previously (Lies et al. 1998).

(Promega, Madison, WI) was added to the recovered RNA. Reverse transcription (RT)-PCR was performed using the RET-ROscript first-strand synthesis kit (Ambion, Austin, TX). Primer p3 (5'-TCT TCA TCG TGT GCA AGG GC-3') was used to synthesize first-strand cDNA from wild-type and bimA10 RNAs. PCR amplification of the cDNA region immediately surrounding intron 3 was performed using Vent DNA polymerase and primers p3 and p1 (5'-AGA TGC TCC GTG ACA AGG GA-3').

**Cell-cycle checkpoint assays:** The procedures of Lies et al. (1998) were used to determine if bimA10 alters the G2 checkpoint preventing entry into mitosis in the absence of NIMA function. nimA-alc mutants contain alcA::nimA as their only copy of nimA, such that germination in glucose medium results in arrest at an APC/C-dependent G2 checkpoint.

To determine if bimA10 alters the checkpoint delaying mitosis in the presence of HU, coverslip cultures containing conidia from ATW53 (bimA10/bimA10) or A852 (diploid, wild-type control) were placed at 42° or 30° for 4 hr in CM media containing both 10 mm HU and 5 μg/ml benomyl or containing only 5 μg/ml benomyl. Benomyl was added to the cultures so that cells would become trapped in mitosis for a period of time and thus, enable an accurate determination of when cells entered mitosis on the basis of %CM1 (Ye et al. 1996).

To determine if bimA10 alters the checkpoint that restrains spindle formation in the presence of HU, ATW53 and A852 conidia were germinated in coverslip cultures at 30° for 10 hr. The cultures were then split and incubated in fresh medium either with or without 10 mm HU at 30°. Samples were collected at 0, 0.5, 1, 1.5, and 2 hr and processed for immunofluorescence analysis to assay for the presence of mitotic spindles.

To determine if bimA10 affects recovery from a slowed S phase, genetically marked control and bimA10 diploids (ATW64 and 65; see Table 1) were germinated in the presence of HU. After 10 hr of growth at 30° in a shake-flask suspension culture, germlings were collected by centrifugation, washed with sterile water, and resuspended by vortexing in 1 ml sterile water. The germlings were plated on CM-agar medium at 30°, and the resultant colonies were monitored for evidence of mitotic recombination according to Kafer (1977).

**RESULTS**

**sepII** is an allele of **bimA**<sup>APC3</sup>: sepII1 was identified in a screen for ts septation mutants (Harris et al. 1994). At the restrictive temperature, the majority of sepII1 germ-lings fail to septate despite having undergone multiple nuclear divisions in the presence of an extending germ tube. All associated phenotypes of sepII1 resulted from a single recessive mutation (data not shown). sepII1 was
found to be linked to proA on chromosome I (data not shown). A chromosome I-specific library (Brody et al. 1991) was used to complement the ts growth of spl1. Cosmid L20D04 was able to rescue all associated spl1 phenotypes (data not shown). Sequence analysis of a complementing SacI fragment from the cosmid identified the 3′ region of bimA<sub>APC3</sub> (O’Donnell et al. 1991; data not shown), suggesting that spl1<sup>+</sup> is bimA<sub>APC3</sub>.

Results from three experiments demonstrated that spl1 is an allele of bimA<sub>APC3</sub>. First, heterozygous diploids with the genotypes sepI<sup>+</sup>/bimA9 and sepI<sup>+</sup>/bimA9 were ts for growth, where bimA1 and bimA9 are ts, recessive mutations (Morris 1976). Second, transformation of sepI strains with a plasmid containing the bimA<sub>APC3</sub> cDNA complemented all sepI<sup>+</sup>-associated phenotypes (data not shown). Third, the sepI mutant contains a mutation in bimA<sub>APC3</sub> that is predicted to dramatically alter BIM<sub>APC3</sub> structure (see below). These results indicate that sepI is an allele of bimA<sub>APC3</sub> and, therefore, we have renamed it bimA10.

**Sequence analyses of bimA1, bimA9, and bimA10:** To investigate the molecular basis of the bimA<sub>APC3</sub> mutant phenotypes, we sequenced genomic clones of bimA<sub>APC3</sub> and the bimA1, bimA9, and bimA10 mutant alleles. bimA<sub>APC3</sub> encodes an 806-amino-acid polypeptide containing 10 copies of a degenerate, 34-amino-acid sequence termed the tetratripeptide repeat (TPR; Sikorski et al. 1990; O’Donnell et al. 1991). The organization of the TPRs is highly conserved between fungi and humans (Figure 1A; adapted from Tugendreich et al. 1993). We found that bimA<sub>APC3</sub> contains three introns: two between TPRs 0 and 1, and one at the end of TPR 9 (Figure 1B).

The bimA1 allele contained T in place of the G in the first base of codon 690, and it directs the replacement of the conserved glycine in TPR 7 with cysteine (Figure 1C). The location of the mutation in bimA1 is in agreement with complementation experiments that positioned it in one of the carboxy-terminal TPR units (O’Donnell et al. 1991). The bimA9 allele contained C in place of the T in the second base of codon 136 in TPR 0 (Figure 1C). This mutation directed the replacement of leucine, a conserved hydrophobic residue, with the hydrophilic residue serine.

Sequencing of two independent genomic clones did not reveal the presence of a mutation in the coding region of bimA10. Analysis of intron splice sites showed that a G-to-A transition in the acceptor site of intron 3 had occurred (Figure 1, B and C). RT-PCR analyses of mRNA from wild-type and bimA10 mutants demonstrated that this mutation did not abolish the splicing of intron 3 (data not shown). Sequence analysis of a C-terminal portion of two independent bimA10 cDNA clones revealed that an alternative acceptor site containing the first G of codon 784 is used. Splicing of intron 3 in bimA10 truncates the remaining coding region by one base and causes a frameshift that is predicted to considerably alter the C terminus of BIM<sub>APC3</sub>. The 23 C-terminal residues, 4 of which belong to the 3′ end of TPR 9, would be replaced with 63 new residues (Figure 1C). The predicted pI of the 23 residues that are removed is 3.4, whereas that of the 63 residues added is 10.4. This splicing error occurs at 30° and, therefore, is not temperature sensitive (data not shown).

**bimA<sub>APC3</sub> mutants have polarity, nuclear division, and chromosome segregation defects:** We phenotypically compared the bimA1, bimA9, and bimA10 mutants. Although all three bimA<sub>APC3</sub> alleles are ts lethal mutations that caused at least a 90% loss in viability after 10 hr incubation at 42° (data not shown), each allele caused cells to arrest growth with distinctive, aberrant cellular morphologies (Figure 2; Table 2). bimA1 germlings (B and C) did not extend germ tubes, unlike wild-type (A), bimA9 (D–F) and bimA10 (G and H) germlings. Calcofluor was used in combination with Hoechst to observe septa, cell walls, and nuclei. Both bimA9 (data not shown) and bimA10 (Figure 2H) germlings displayed septation defects (Harris et al. 1994; see Figure 7) as well as diffuse calcofluor-staining bands along their germ tubes, demonstrating that these mutants inappropriately deposit cell wall material. In addition, bimA10 germlings were irregularly shaped, containing swollen regions and unusual branching. The differential effects of these bimA<sub>APC3</sub> alleles on germling morphology suggest that the APC/C may play a role in the establishment or maintenance of polar growth in A. nidulans.

The terminal arrest morphology and spacing of nuclei also differed among bimA<sub>APC3</sub> mutants (Figure 2). Wild-type germlings (A) undergo synchronous nuclear divisions (Clutterbuck 1970) and are usually found with interphase nuclei, which contain spherical, non-Hoechst-staining nucleoli. Mitotic nuclei are smaller, do not have obvious nucleoli and stain more intensely. Wild-type germling 1 in Figure 2 has completed two nuclear divisions, while germlings 2 and 3 have completed three nuclear divisions. The nuclei of germlings 1 and 2 are arranged in pairs and are well separated from each other. Each pair represents daughter nuclei from the preceding nuclear divisions (Suelmann et al. 1997). Germling 3 has eight nuclei, five of which are evenly spaced along the germ tube. A septum is visible at the base of the germ tube in this cell.

Abnormal chromatin masses and mitotic nuclei were commonly observed in bimA<sub>APC3</sub> mutants. bimA10 germlings frequently arrested growth with one or two condensed nuclei (Figure 2, B and C; Table 2). Incubation at a slightly higher temperature resulted in a mitotic block during the first division (O’Donnell et al. 1991). Nuclear structure and distribution were abnormal in bimA9 (D–F) and bimA10 (G and H) mutants, with some cells containing mitotic nuclei, some containing interphase nuclei, and other cells containing both interphase and mitotic nuclei. This latter result shows that nuclei in some bimA9 and bimA10 cells underwent...
Hypomorphic bimA<sup>APC3</sup> Alleles

asynchronous divisions even though they were in a common cytoplasm. Unlike bimA1, the arrest phenotypes of bimA9 and bimA10 strains were not affected by increasing the incubation temperature (data not shown).

Mitotic progression of bimA1, bimA9, and bimA10 germlings was monitored (Figure 3; Table 2). After a 3-hr germination period, the wild-type control underwent a nuclear division approximately every hour (Figure 3A; Table 2). Half of the bimA1 germlings were able to execute one mitotic division with kinetics slower than the control, but they were unable to divide further (Figure 3A; Table 2). Many bimA1 cells accumulated a morphologically abnormal chromatin mass after 7 hr at the restrictive temperature. Both bimA9 and bimA10 mutants underwent their first nuclear division with kinetics similar to the control, but they progressively became more delayed in nuclear division. Like those of bimA1, many nuclei of bimA9 and bimA10 cells were morphologically abnormal (see Figures 2 and 4). Interestingly, we observed a population of bimA10 germlings with nuclear division kinetics faster than those of the wild-type control (Table 2).

We calculated the percentage of mitotic cells (%CMI) in cultures of bimA<sup>APC3</sup> mutants to determine if the nuclear division delays occurred during mitosis (Figure 3B). The %CMI of the control strain ranged between 2 and 5%. bimA1 germlings accumulated an elevated %CMI. The 38% peak at 240 min represents the delay bimA1 encountered during the first mitosis, while the peak at 300 min represents the delay encountered during entry into a second mitosis. bimA10 germlings did not exit this second mitosis successfully, because germlings with four nuclei were not observed (Table 2). The majority of bimA9 and bimA10 germlings entered the first and second mitoses at ~220 and 280 min, and progression through these mitoses was delayed (Figure 3B).

bimA10 germlings were stained with Hoechst and anti-tubulin antibodies to further characterize the nuclear division defects (Figure 4). Figure 4, A and B, shows a
Figure 2.—Cellular morphologies of $\text{bimA}^+$ and $\text{bimA}$ germlings. Conidia from strains A28 ($\text{bimA}^+$), PM156 ($\text{bimA}^+$), MLC1-19 ($\text{bimA}$), and ATW41 ($\text{bimA}$) were germinated on coverslips in complete media at 42°C. Coverslips were removed at various intervals, fixed, and stained. (A) A28 was fixed after 7 hr and stained with Hoechst 33258 and Calcofluor. (B and C) PM156 was fixed after 9 hr and stained with Hoechst 33258. (D) MLC1-19 was fixed after 7 hr or (E and F) 9 hr and stained with Hoechst 33258. (G) ATW41 was fixed after 9 hr and stained with Hoechst 33258 or (H) Hoechst 33258 and Calcofluor. To demonstrate abnormal nuclear division and morphology, arrowheads point to (D) nuclear clumping, (E and F) condensed chromatin, and (G) an anaphase bridge of chromatin. Bars, 5 μm.

The anaphase bridges revealed by antitubulin staining suggested that chromosome segregation was defective in $\text{bimA}$ mutants. We employed a genetic assay to test for chromosome segregation defects in $\text{bimA}$, $\text{bimA}$, and $\text{bimA}$ germlings (Figure 5). Strains were germinated at 42°C for 10 hr and then placed at the permissive temperature. Under these conditions, a small percent of $\text{bimA}$ germlings survive. Formation of aneuploid colonies by this surviving population of germlings would indicate that defective chromosome segregation had occurred at the restrictive temperature. Identification of aneuploid colonies is easy because of their characteristic abnormal morphology (Kafer and Upshall 1973). The $\text{sepB3}$ mutant has previously been shown to affect chromosome segregation in this and other assays (Harris and Hamer 1995) and so was used in this experiment as a control. Figure 5 shows euploid and aneuploid colonies formed after the shift-down experiment. Approximately 20% (83/398) of $\text{bimA}$, 28% (32/112) of $\text{bimA}$, 12% (17/138) of $\text{bimA}$, 24% (37/152) of $\text{sepB3}$, and 0% (0/200) of $\text{bimA}^+$ surviving germlings gave rise to aneuploid colonies. Therefore, chromosome segregation is defective in $\text{bimA}_{\text{APC3}}$ mutants.

In addition to being required for completion of mitosis, $\text{bimA}_{\text{APC3}}$ is also required in late G2 to prevent premature entry into mitosis when the NIMA kinase is defective (Lies et al. 1998; Ye et al. 1998). To determine if $\text{bimA}$ affects $\text{bimA}_{\text{APC3}}$ function in this G2 checkpoint, we constructed $\text{nimA}$, $\text{bimA}$ double mutants and as-
Hypomorphic bimA<sup>APC3</sup> Alleles

### TABLE 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>bimA&lt;sup&gt;+&lt;/sup&gt;</th>
<th>bimA10</th>
<th>bimA9</th>
<th>bimA10</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 min</td>
<td>85</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>33</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>300 min</td>
<td>1</td>
<td>36</td>
<td>62</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>88</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>36</td>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>420 min</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8</td>
<td>52</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>19</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>540 min</td>
<td>n/a</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>15</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7</td>
<td>9</td>
<td>28</td>
</tr>
</tbody>
</table>

**a** Number of nuclei in the germtube.

**b** Morphologically abnormal chromatin mass (see Figures 2 and 4).

...sayed for entry into mitosis during spore germination under restrictive conditions for both nimA and bimA10. Figure 6 shows the results for nimA-alc, bimA<sup>APC3</sup> double mutants, where nimA-alc is an allele of nimA in which nimA expression is repressed by glucose (Lies et al. 1998). Whereas nimA-alc single mutants fail to enter mitosis, up to 50% of the cells of a nimA-alc, bimA10 double mutant enter mitosis with kinetics indistinguishable from the nimA-alc, bimA1 or nimA-alc, bimA9 double mutants (Figure 6) or the wild-type control (data not shown). Similar results were obtained for double mutants containing bimA10 in combination with the ts nimA5 allele (data not shown). Thus, each mutant is severely defective for the G2 checkpoint function of bimA<sup>APC3</sup>.

Mutations in DNA damage checkpoint genes allow septation to proceed in bimA9 and bimA10 germlings: Given that the APC/C is required for the metaphase-to-anaphase and M-to-G1 transitions, we expected to observe chromosome segregation abnormalities and elevated %CMIIs in the bimA1, bimA9, and bimA10 strains. However, the aseptate phenotype of bimA9 and bimA10 (Harris et al. 1994; see below) implied a novel role for the APC/C in septation control. Alternatively, errors in DNA metabolism caused by the mutations may lead to the observed septation block via the A. nidulans DNA damage checkpoint.

**Figure 3.—Mitotic progression in bimA mutants.** (A) Nuclear division kinetics and (B) %CMI of bimA mutants. Conidia from strains A28 (bimA<sup>+</sup>), PM156 (bimA1), MLC1-19 (bimA9), and ATW41 (bimA10) were germinated on coverslips in complete media at 42°. At various time intervals, coverslips were removed, fixed, and stained with Hoechst 33258 to observe nuclei. Each data point was determined using 100 germlings.
Harris and Kraus (1998) have shown that components of the DNA damage checkpoint in *A. nidulans*, such as *uvsB* and *uvsD* (Ye et al. 1997a), delay or prevent septum formation when DNA metabolism is perturbed. It was recently discovered that *uvsB* is a homologue of the *MEC1/rad3* PI-3-related kinases involved in the DNA damage checkpoint in yeasts (Seaton et al. 1992; Katô and Ogawa 1994; S. Harris, personal communication). Thus, the failure of *bimA9* and *bimA10* mutants to septate could be explained by the accumulation of DNA metabolism errors.

To test if the failure of *bimA9* and *bimA10* germlings to septate was a result of the activity of the DNA damage checkpoint, *uvsB110* and *uvsD153* mutations were crossed into *bimA9* and *bimA10* strains. We found that the *uvs* mutations partially relieved the septation defects.

**Figure 4.** *bimA10* affects both the synchrony and fidelity of mitosis. A28 (*bimA1*; A–D) and ATW41 (*bimA10*; E–J) were germinated on coverslips for 9 hr at 42°C. Samples were then fixed and stained with Hoechst 33258 (left panels) and a mouse anti-α-tubulin antibody (right panels). The arrowhead in E points to chromatin that is spread across an anaphase spindle. Bar, 5 μm.
Hypomorphic bimAPC3 Alleles

Figure 6.—bimA10 is defective for the G2 checkpoint, which blocks entry into mitosis when NIMA expression is deficient. The NIMA kinase is required for entry into mitosis. In the absence of normal NIMA function, BIMA restrains mitosis. bimA alleles were crossed into a strain with a single nimA+ kinase gene under the control of the glucose-repressible, ethanol-inducible alcA promoter. Coverslip cultures were incubated at 43°C fixed, and stained with Hoechst 33258. For each data point, the %CMI was determined by measuring the percentage of germlings (N > 300) with condensed chromatin arrays. Arrowheads point to typical aneuploid colonies, and the asterisk is positioned next to a haploid colony. (ATW41); *, nimA-alc, bimA10 (SFC552-28).

Figure 5.—The bimA mutations cause defects in chromosome segregation. Conidia were spread onto CM plates and incubated for 10 hr at 42°C. Plates were then placed at 30°C for 3 days. (A) ASH60 (sepB3), (B) PM156 (bimA1), (C) MLC1-19 (bimA9), and (D) ATW41 (bimA10). Chromosome loss is indicated by the production of aneuploid colonies that are small and may contain haploid sectors that spread in radial arrays. Arrowheads point to typical aneuploid colonies, and the asterisk is positioned next to a haploid colony.

at the restrictive temperature (Figure 7, A and B), consistent with an indirect effect of bimA9 and bimA10 on septation.

The DNA damage checkpoint is critical for cell survival after genomic insult; mutations that cause DNA damage commonly produce synthetic phenotypes in DNA damage checkpoint-deficient backgrounds. We observed that the bimA10, uvsB110 and bimA10, uvsD153 double mutants displayed pronounced growth defects at 30°C, while the bimA9, uvsB110 and bimA9, uvsD153 double mutants displayed growth defects at 37°C (Figure 7C). In addition to growth defects, the uvsB110 and uvsD153 alleles also enhanced the mitotic defects of the bimA9 and bimA10 strains (data not shown). Taken together, our results suggest that bimA9 and bimA10 mutants accumulate errors in DNA metabolism that activate the DNA damage checkpoint blocking septation.

bimA10 is hypersensitive to hydroxyurea: Hypersensitivity to HU is associated with defects in cell-cycle processes and checkpoint regulation (Ye et al. 1996). We observed that both bimA1 and bimA9 alleles conferred slight sensitivities to low concentrations of HU (8 mm) at permissive temperature (Figure 8A). The sensitivity of bimA10 mutants was more striking (Figure 8A). HU sensitivity cosegregated with the bimA ts phenotypes and was complemented with the bimAPC3 cDNA (data not shown), presumably through gene conversion.

Hypersensitivity to HU is characteristic of S-phase checkpoint mutants of A. nidulans (Ye et al. 1996). We tested whether the HU hypersensitivity of bimA10 was a checkpoint defect by examining if mitosis was delayed in the presence of HU. We found that like the wild type, bimA10 mutants were able to delay both chromatin condensation (Figure 8B) and spindle assembly (data not shown) when treated with 10 mm HU. In addition, we did not observe increased mitotic recombination in bimA10 mutants after HU treatment (data not shown), suggesting that the ability to recover from a slow S phase was normal (Stewart et al. 1997). We conclude that bimA10-associated HU hypersensitivity is not due to a faulty S-phase checkpoint, and may instead be the result of a defect associated with S-phase regulation.

DISCUSSION

We found that sep1 is an allele of bimAPC3 (and so, was renamed bimA10), possibly implicating BIMAAPC3 in the control of septation. Septation defects and hypomorphic phenotypes are associated with both bimA9 and bimA10. The results shown here demonstrate that the sep phenotype of bimA9 and bimA10 is at least partially caused by the activation of a DNA damage checkpoint, suggesting that mutations in bimAPC3 can lead to errors in DNA metabolism. This idea is supported by our observations that bimA9 and bimA10 strains have morphologically abnormal nuclei, chromosome segregation defects, synthetic phenotypes with mutations in the DNA
damage checkpoint genes uvsB^{MEC1/rad3} or uvsD^{+}, and enhanced sensitivity to hydroxyurea.

The molecular basis of bimA^{APC3} alleles: Sequencing of bimA1 and bimA9 revealed that each allele contained a single amino acid substitution in an important TPR residue (Figure 1: bimA1, G to C in TPR 7; bimA9, L to S in TPR 0). TPR units are known to mediate protein-protein interactions (Goebl and Yanagida 1991; Sikorski et al. 1991; Lamb et al. 1995). Crystallographic studies of the TPR protein PP5 and modeling of Nuc2 protein structure support the protein-protein interaction model and point out that adjacent TPR units pack together in an arrangement of antiparallel α helices that make a potential binding site for target proteins (Hirano et al. 1990; Das et al. 1998). The bimA1 and bimA9 mutations may destabilize protein-protein interactions within BIMA^{APC3}, between BIMA^{APC3} and other APC/C components, or between BIMA^{APC3} and target proteins. A bimA1-like substitution in TPR 6 of the bimA^{APC3} homologue, CDC27 of S. cerevisiae, destabilizes the interaction between Cdc27p and another APC/C component, Cdc23p (Lamb et al. 1994). Perhaps the difference in the bimA1 and bimA9 phenotypes results from the interaction of TPRs 0 and 7 with different proteins.

The intron splicing error of bimA10 alters BIMA^{APC3} considerably by destroying the end of TPR 9, adding 40 C-terminal residues, and changing the charge of the C terminus from acidic to basic (Figure 1). Because the splicing error is not temperature sensitive, this allele most likely encodes a thermosensitive polypeptide. The similarity between bimA9 and bimA10 mutants may reflect a similar function for TPRs 0 and 9.

bimA mutants have defects that activate a DNA damage checkpoint blocking septation: Although bimA1, bimA9, and bimA10 had quantitatively different effects on cell growth and nuclear division, all three bimA alleles conferred defects in mitotic progression, chromosome segregation, and the G2 checkpoint preventing mitosis in response to deficient NIMA. Similar phenotypes are associated with mutations in the APC/C subunits BIME^{APC1} and BIMH^{APC6} (Lies et al. 1998; P. M. Mira-bit o, unpublished results), making it likely that the three bimA mutations affect one or more functions of the APC/C itself, rather than some unknown, APC/C-independent functions of BIMA^{APC3}.

The septation phenotype of bimA9 and bimA10 mu-

Figure 7.—The uvs mutations allow septum formation in bimA9 and bimA10. (A) Conidia from strains A28 (bimA^{+}), MLC1-19 (bimA9), ATW41 (bimA10), ATW55 (bimA9 uvsD153), ATW56 (bimA10 uvsD153), ATW57 (bimA10 uvsB110), and ATW66 (bimA9 uvsB110) were germinated on coverslips in complete media at 42° for 10 hr. Coverslips were then fixed and stained with Hoechst 33258 and Calcofluor. Percentage of germlings (N = 100) with septa was determined. Only germlings with at least eight nuclei in a germtube 70 μm or longer were considered. (B) Examples of bimA10 uvsD153 germlings with septa (ATW56 is shown). Bar, 5 μm. (C) Genetic interactions between bimA and uvs alleles. Conidia were point inoculated onto CM plates and incubated at 30° or 37° for 3 days (from left to right): Row 1: MLC1-19 (bimA9) and ATW41 (bimA10). Row 2: ASH201 (uvsB110), ASH206 (uvsD153), and ATW66 (bimA9 uvsB110). Row 3: ATW55 (bimA9 uvsD153), ATW57 (bimA10 uvsB110), and ATW56 (bimA10 uvsD153).
Hypomorphic bimA APC3 Alleles initially suggested a role for the APC/C in regulating septation in A. nidulans. This was a particularly attractive hypothesis, as the phenotypes from loss-of-function mutations and overexpression of the Schizosaccharomyces pombe bimA APC3 homologue nuc2 suggested that nuc2 may be a negative regulator of septation (Hirano et al. 1988; Kumada et al. 1995). Our results do not, however, support a direct role for bimA APC3 as either a positive or negative regulator of septation. Instead, we find that the septation defect of bimA9 and bimA10 mutants was largely dependent on a DNA damage checkpoint that blocks septation. This checkpoint has previously been shown to block septation in the presence of low levels of genotoxic agents and in the sepB3 and sepJ1 mutants (Harris and Kraus 1998). The fact that not all bimA9 and bimA10 cells formed septa upon inactivation of the DNA damage checkpoint may result from the synthetic interactions among these bimA APC3 and uvs alleles (see Figure 7C). It is unlikely that chromosome missegregation alone is the cause of the septation block, because aneuploid A. nidulans strains can septate (Wolkow et al. 1996). We suggest that bimA9 and bimA10 mutants accumulate errors in DNA metabolism and that their septation defect is a secondary consequence of these errors.

Suggestions for how bimA APC3 mutants give rise to DNA metabolism errors: One clue to the mechanism may be provided by the extreme HU-sensitive phenotype of bimA10 mutants (Figure 8A). This HU hypersensitivity is not caused by defects in the S-phase checkpoint controlling entry into mitosis (Figure 8B) or in the pathway controlling recovery from S-phase perturbation (data not shown), suggesting that the APC/C may be more intimately involved in DNA replication. Additional evidence for APC/C involvement in replication comes from observations that mutations in the APC/C genes CDC27 and CDC16 cause uncontrolled replication in S. cerevisiae (Heichman and Roberts 1996, 1998). Perhaps inappropriate initiation of replication occurs at some level in bimA9 and bimA10 mutants, leading to partially overreplicated chromosomes that may activate the DNA damage checkpoint blocking septation.

Ye et al. (1998) recently suggested a novel role for bimA APC3 in mitotic regulation. Rapid temperature shift with the bimA1 strain promoted repeating cell-cycle oscillations (chromosome condensation and decondensation, and activation and inactivation of mitotic kinases) devoid of intermittent nuclear divisions. The authors suggest that bimA APC3 is part of a "cell-cycle clock mechanism" that coordinates APC/C function with the activity of mitotic cell-cycle regulators (p34cdc2, NIMA). In this way, the bimA1 mutation may cause mitotic delay by desensitizing the APC/C to activation by its mitotic substrates (cyclin B, Polo, NIMA). The complete inability of bimA1 to regulate APC/C dependent-proteolysis of an anaphase inhibitor(s) may account for the lack of nuclear division in the mutant.

Figure 8.—bimA10 hypersensitivity to hydroxyurea is not caused by a defective S-phase checkpoint. (A) bimA10 hypersensitivity to hydroxyurea. Conidia from strains A28 (bimA1), PM156 (bimA1), MLC1-19 (bimA9), and ATW41 (bimA10) were spread onto CM plates containing 8 mM HU. Pictures were taken after 6 days of growth at 30°C. The differential shading among colonies is caused by the different color markers in each strain, where WT is green, bimA1 is white, and bimA9 is yellow. Furthermore, conidiation of bimA1 and bimA9 colonies is reduced by growth on these media. (B) bimA10 delays chromatin condensation in the presence of HU. Conidia of A852 (bimA+/bimA1) and ATW53 (bimA10/bimA10) were germinated at 42°C for various lengths of time in the presence or absence of 10 mM HU. Samples were fixed and stained with Hoechst 33258. At each time point, the percentage of germings (N = 100) with a condensed nucleus was determined (%CMI). Benomyl (5 μg/ml) was added to the cultures to trap nuclei in mitosis to better determine the rate at which nuclei entered mitosis (Ye et al. 1996). Similar results were obtained at the permissive temperature of 30°C (data not shown).
To extend this hypothesis, bimA9 and bimA10 may differ from bimA1 in the ability to promote the metaphase-to-anaphase transition, thus allowing some extent of nuclear division to occur. The asynchronous nuclear divisions and chromosome segregation abnormalities of bimA9 and bimA10 cells are possibly caused by inefficient APC/C-dependent proteolysis of an anaphase inhibitor(s). Perhaps this problem leads to the production of daughter nuclei joined by anaphase bridges of chromatin (see Figure 4, E and F). We envision that the production of disorganized chromatin masses with multiple spindles occurs after these nondisjoined daughter nuclei enter the next round of mitosis, similar to that seen in bimB ESP1 mutants (May et al. 1992). Alternatively, this observation may suggest that bimA APC/C helps coordinate spindle pole body duplication with the nuclear division cycle.

We thank Dr. John Clutterbuck for providing pDHG25 and Dr. Steve Harris for providing uvs strains, communicating results prior to publication, and for many insightful suggestions. We also thank Dr. John Doonan and members of the Mirabito and Hamer labs for helpful discussions, as well as the Genetics reviewers for their constructive criticisms. This work was funded by two National Institutes of Health grants awarded to J.E.H. and P.M.M.

LITERATURE CITED


Irniger, S., and K. Nasmyth, 1997 The anaphase-promoting complex is required in G1 arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase. J. Cell Sci. 110: 1523-1531.


Momany, M., and J. E. Hamer, 1997 The relationship of actin,
Hypomorphic bimA^{APC3} Alleles


Communicating editor: R. H. Davis