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Checkpoint Defects Leading to Premature Mitosis Also Cause Endoreplication of DNA in *Aspergillus nidulans*

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The G2 DNA damage and slowing of S-phase checkpoints over mitosis function through tyrosine phosphorylation of $NIMX^{cdc2}$ in *Aspergillus nidulans*. We demonstrate that breaking these checkpoints leads to a defective premature mitosis followed by dramatic rereplication of genomic DNA. Two additional checkpoint functions, *uvsB* and *uvsD*, also cause the rereplication phenotype after their mutation allows premature mitosis in the presence of low concentrations of hydroxyurea. *uvsB* is shown to encode a *rad3/ATR* homologue, whereas *uvsD* displays homology to *rad26*, which has only previously been identified in *Schizosaccharomyces pombe*. $uvsB^{rad3}$ and $uvsD^{rad26}$ have G2 checkpoint functions over mitosis and another function essential for surviving DNA damage. The rereplication phenotype is accompanied by lack of $NIME^{cyclinB}$, but ectopic expression of active nondegradable $NIME^{cyclinB}$ does not arrest DNA rereplication. DNA rereplication can also be induced in cells that enter mitosis prematurely because of lack of tyrosine phosphorylation of $NIMX^{cdc2}$ and impaired anaphase-promoting complex function. The data demonstrate that lack of checkpoint control over mitosis can secondarily cause defects in the checkpoint system that prevents DNA rereplication in the absence of mitosis. This defines a new mechanism by which endoreplication of DNA can be triggered and maintained in eukaryotic cells.

INTRODUCTION

Checkpoint pathways have been identified that respond to damaged or incompletely replicated DNA to prevent cell cycle progression and to subsequently allow DNA repair or the completion of replication (Elledge, 1996; Nurse, 1997; Weinert, 1997, 1998). The regulation of tyrosine 15 phosphorylation of the cdc2 kinase plays a crucial role in the control of mitotic entry. During interphase, cdc2 associates with its cyclin partner cyclin B (Evans *et al.*, 1983; Booher *et al.*, 1989), but the complex is kept inactive by phosphorylation at tyrosine 15 of cdc2 by *Wee1/Mik1/Myt1* inhibitory tyrosine kinases (Gould and Nurse, 1989; Lundgren *et al.*, 1991; Mueller *et al.*, 1995). At the G2–M transition, cdc2/cyclin B is rapidly activated by tyrosine 15 dephosphorylation carried out by the cdc25 phosphatase (Russell and Nurse 1986; Gould and Nurse, 1989). The inability to phosphorylate the inhibitory tyrosine residue of cdc2 results in premature mitosis in many model systems (Gould and Nurse 1989; Broek *et al.*, 1991; Krek and Nigg, 1991; Hayles *et al.*, 1994; Blasina *et al.*, 1997), including *Aspergillus nidulans* (Ye *et al.*, 1997b), but not in *Saccharomyces cerevisiae* (Amon *et al.*, 1992; Sorger and Murray, 1992). Lack of tyrosine phosphorylation of cdc2 abolishes the slowed S-phase and G2 DNA damage checkpoints in *A. nidulans*, which prevents entry into mitosis in the presence of incompletely replicated or damaged DNA, respectively (Ye *et al.*, 1996, 1997b). This mechanism of delaying mitotic entry is conserved in *Schizosaccharomyces pombe* and mammalian systems (Jin *et al.*, 1996; Rhind *et al.*, 1997), as are many of the upstream regulators of these pathways.

In *A. nidulans*, BIME has also been demonstrated to play a role in the control of the S-phase checkpoint (Ye *et al.*, 1996). *bimE* is an anaphase-promoting complex 1 (APC1) homologue and was originally identified as being required for exit from mitosis (Morris, 1976). Although the absence of cdc2 tyrosine phosphorylation is sufficient to allow mitosis in the presence of low concentrations of hydroxyurea (HU), these cells are not able to overcome a complete S-phase arrest in the presence of high concentrations of HU (Ye *et al.*, 1996). However, lack of cdc2 tyrosine phosphorylation in combination with compromised BIME function is sufficient to overcome this S-phase arrest (Ye *et al.*, 1996). This appears to be regulated through the mitosis-promoting NIMA kinase, because lack of cdc2 tyrosine phosphorylation allows the accumulation of NIMA protein during S-phase arrest, and inactivation of BIME leads to the activation of NIMA by phosphorylation (Ye *et al.*, 1996).

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In addition to checkpoint systems that ensure the completion of DNA replication or DNA repair before entry into mitosis, cells also have mechanisms that restrict DNA replication to occurring only once per cell cycle (Stillman, 1996; Wuarin and Nurse, 1996). This single round of replication is followed by mitosis, resulting in the segregation of the duplicated chromosomal DNA. Only once mitosis is completed can cells enter S-phase and replicate DNA again. Components of the cell cycle regulatory machinery are involved in maintaining this temporal order, which ensures the maintenance of genome ploidy (Hayles *et al*., 1994; Moreno and Nurse, 1994; Nasmyth, 1996; Nishitani and Nurse, 1997).

We were interested in identifying upstream regulators of tyrosine phosphorylation of the *A. nidulans* NIMX^{cdc2} kinase. Many DNA damage-sensitive mutants have been identified in *A. nidulans* (Jansen, 1970; Kafer and Mayor, 1986; Kafer and Chae, 1994; Kafer and May, 1997; Zhao and Kafer, 1992; Osman *et al*., 1993; Yoon *et al*., 1995; van Heemst *et al*., 1997; Han *et al*., 1998). Of these, *uvsH* encodes a DNA repair gene with homology to *S. cerevisiae* RAD18 (Yoon *et al*., 1995), *uvsC* is an *S. cerevisiae* RAD51 homologue (van Heemst *et al*., 1997), *uvsI* is an *S. cerevisiae* REV3 homologue (Han *et al*., 1998), and *uvsF* displays homology to DNA replication factor C (Kafer and May, 1997). In addition to having roles in the DNA damage repair response, some of these DNA damage-sensitive mutants are likely to have roles in checkpoint regulation. To identify these genes, we screened the known *A. nidulans* DNA damage-sensitive mutants for sensitivity to low concentrations of HU. Strains which are sensitive to both HU and DNA damage are likely candidates for having roles in checkpoint responses to G2-DNA damage and slowed S-phase. Here we describe the identification and complementation of two of these genes. *uvsB* encodes a *rad3* homologue, whereas *uvsD* displays sequence and structural similarity to *rad26* and is likely to be the first identified *rad26* homologue. Similar to mutations that prevent tyrosine 15 phosphorylation of NIMX^{cdc2}, mutations in *uvsB* or *uvsD* result in loss of checkpoint regulation in response to G2-DNA damage or prolonged S-phase. Moreover, loss of checkpoint regulation in these mutants in the presence of low concentrations of HU leads to a dramatic rereplication phenotype characterized by highly polyploid nuclei. Cells displaying the rereplication phenotype have low NIMX^{cdc2} kinase activity because of loss of NIME^{cyclinB}, but ectopic expression of nondegradable NIME^{cyclinB} does not prevent DNA rereplication even though NIMX^{cdc2} kinase activity is maintained at a high level. We propose that loss of checkpoint regulation over mitosis can secondarily cause defects in mechanisms that prevent DNA rereplication in the absence of mitosis.

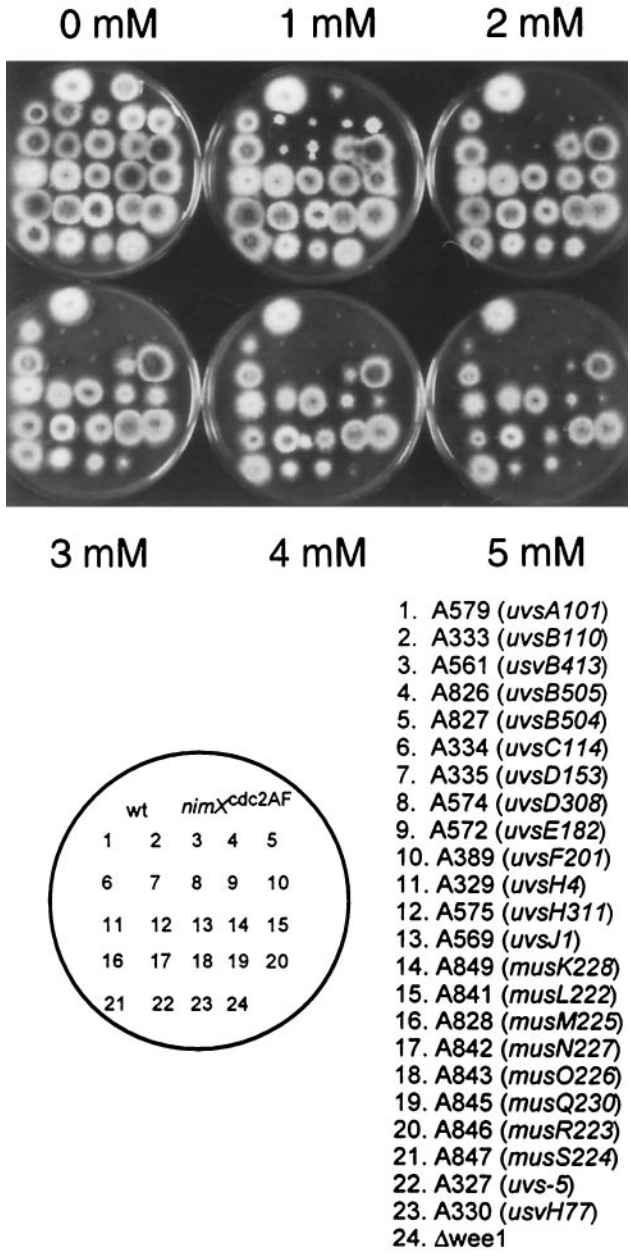

Figure 1. Mutations in *uvsB* and *uvsD* cause marked HU sensitivity. The indicated strains were replica plated onto media containing varying concentrations of HU. After incubation plates were photographed.

MATERIALS AND METHODS

A. nidulans Strains and General Techniques

A. nidulans strains used in this study were R153 (*pyroA*4; *wA*3); GR5 (*pyrG*89; *pyroA*4; *wA*3); ΔankA^{wee1} (Δ*ankA*^{wee1}; pyrG89; *pyr*4+; *pyroA*4; *wA*3); FRY20-1 (*nimX*^{cdc2AF}; *pyroA*4; *pyrG*89; *wA*3); AT27 (*nimX*^{cdc2AF}; *nimA*5; *pyroA*1; *riboA*2; *wA*3); SO54 (*nimA*5, *wA*2); AT158 (*uvsD*308; *nimA*5; *riboA*1, *fwA*); AT136 (*uvsB*505; *nimA*5; *nic*A2); AT103 (*uvsD*308; *pyrG*89; *riboA*1, *pyroA*4 *wA*3); AT107 (*uvsB*505; *pyrG*89; *pyroA*4; *chaA*1); A329 (*uvsH*4; *adE*20; *biA*1; *methG*1; *pyroA*4; *wA*3); A826 (*uvsB*505; *choA*1; *biA*1; *chaA*1);A574 (*uvsD*308; *riboA*1; *biA*1; *chaA*1); AT33–1 (*bimE*7; *nimX*^{cdc2AF}; *pyroA*4; *pabaA*1; *pyrG*89); and AT214 (*nimX*^{cdc2AF} + *alcA::nimE*$^{\Delta D}$; *pyroA*4; *pyrG*89 *wA*3). AT214 was generated by transformation of FRY20-1 with the plasmid p122 containing a version of *nimE*, which does not contain the destruction box and which is under control of the *alcA* promoter (*alcA::nimE*$^{cyclin\ B\Delta D}$). Plasmid p122 was a kind gift from Dr. Mathew O'Connell (Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia). The genotypes of strains listed in Figure 1 are available at http://www.kumc.edu/research/fgsc/nidlist.html. Media and general techniques for culture of *A. nidulans*, DAPI staining for chromosome mitotic index, protein extraction, immu-

noprecipitation, protein kinase assays, and Western blotting were as previously described (Osmani *et al.*, 1987, 1991a,b, 1994; Oakley and Osmani, 1993; Ye *et al.*, 1995). Measurements of relative DNA content were made as described by May *et al.* (1992) using an Eclipse E800 (Nikon, Tokyo, Japan) microscope equipped with a digital camera system. The data collected were analyzed with Phase 3 Imaging Systems software (Media Cybernetics, Silver Spring, MD), and the values presented represent the average fluorescence intensity for single nuclei (subtracting background) in 12 cells for each strain.

Library Construction, Aspergillus nidulans Transformation, and Complementation of uvsB505 and uvsD308

Genomic DNA isolated from GR5 was partially digested with *Alu*I to generate blunt-ended gDNA fragments of an average size of ~7 kb. The gDNA (14 μg) was then ligated to an adaptor (28 μg) in a total volume of 40 μl at 16°C overnight in the presence of 5 mM spermidine. The sequence of the 5′ phosphorylated adaptor was as follows: 5′-ATCCGGCACGAG-3′ and 5′-CTCGTGCCG-3′. After ligation, the DNA was precipitated, and adaptor-ligated genomic DNA (gDNA) was separated from unligated linkers by agarose gel electrophoresis. Adaptor-ligated gDNA (4–20 kb) was excised from the gel and purified by freeze–thawing and ethanol precipitation (Qian and Wilkinson, 1991). Adaptor-ligated gDNA was then ligated into pRG3 (Waring *et al.*, 1989), which had been digested with *Bam*HI and partially filled by incubation with *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT) in the presence of 25 μM dGTP for 2 min at 72°C. Ligated DNA was transformed into Epicurian Coli XL10-Gold Ultracompetent cells (Stratagene, La Jolla, CA) or TOP10 Electocomp *Escherichia coli* cells (Invitrogen, San Diego, CA), and transformants were selected by resistance to ampicillin. Ampicillin-resistant colonies (7.7×10^5) were obtained, 80% of which contained inserts of an average insert size of 6.7 kb, giving >170 genomic equivalents assuming a genome size of 2.3×10^4 kb (Timberlake, 1978). Aliquots of the primary library were stored in Luria–Bertani medium containing 20% glycerol at −70°C. The remaining cells were grown overnight at 37°C, and plasmid library DNA was isolated using the alkaline lysis procedure followed by purification on a cesium chloride gradient using standard procedures (Maniatis *et al.*, 1982).

Complementation of *uvsB505* and *uvsD308* alleles was carried out by library transformation of AT107 and AT103, respectively, using standard techniques (Oakley and Osmani, 1993) selecting for transformants that were complemented for sensitivity to HU and methyl methane sulfonate (MMS). Single-copy integration was confirmed by Southern blotting, and plasmids were recovered from complemented strains (Osmani *et al.*, 1987). Recovered plasmids were retransformed into the original mutant strains, and complementing plasmids were sequenced. We confirmed homologous integration at the *uvsB* locus by two-step gene replacement (Osmani *et al.*, 1987) and for *uvsD* by sequencing the mutant *uvsD308* allele. To obtain the coding sequence for *uvsB* and *uvsD*, rapid amplification of cDNA ends (RACE)-PCR was performed using the Marathon cDNA amplification kit (Clontech, Cambridge, United Kingdom), and the 5′ and 3′ RACE-PCR products were sequenced.

Targeted Disruption of uvsB and uvsD

Targeted disruption of *uvsB* was performed using standard techniques (Osmani *et al.*, 1994) by transforming GR5 with a plasmid containing a 6153-bp internal fragment of *uvsB* genomic DNA. Transformants were able to grow in the absence of uridine and uracil and contained the above plasmid integrated homologously at *uvsB*. This leads to a duplication of *uvsB* with one copy lacking its 3′ end and the other lacking its 5′ end. The 3′-deleted version lacks 1815 bp of 3′ coding sequence, including the kinase domain, and also its normal termination and processing sequences. The 5′-deleted version lacks a promoter and 800 bp of the 5′ coding sequence. A similar strategy was use to disrupt *uvsD* using a 1512-bp internal fragment. Homologous integration disrupts *uvsD* generating a 3′-deleted version lacking 465 bp of coding sequence and normal termination and processing sequences and a 5′-deleted version lacking 407 bp of 5′ coding sequence and its promoter. Disruptions were confirmed by Southern blotting.

Sensitivity Test to UV Irradiation and nimA5 Block Release

Nondividing and dividing cells were tested for sensitivity to UV irradiation as previously described using a microprocessor controlled UV cross-linker (FBUVXL-1000; Fischer Biotech, Pittsburgh, PA; 254 nm) (Ye *et al.*, 1997b). Entry into mitosis after MMS (0.02%) treatment at the *nimA*5 arrest point was determined as previously described (Ye *et al.*, 1997b).

RESULTS

uvsB and uvsD Mutants Are Sensitive to Both DNA Damage and Prolonged S-Phase

We have previously demonstrated that checkpoint regulation over entry into mitosis is regulated through tyrosine phosphorylation of the NIMX^{cdc2} kinase in response to G2 DNA damage or prolonged S-phase (Ye *et al.*, 1996, 1997b). To identify upstream regulators of checkpoint regulation of NIMX^{cdc2} tyrosine phosphorylation, we screened known DNA damage-sensitive mutants (Jansen, 1970; Kafer and Mayor, 1986; Kafer and Chae, 1994; Kafer and May, 1997; Zhao and Kafer, 1992; Osman *et al.*, 1993; Yoon *et al.*, 1995; van Heemst *et al.*, 1997; Han *et al.*, 1998) for sensitivity to the DNA replication inhibitor HU (Figure 1). Of the 18 genes tested, all four alleles of *uvsB* and both alleles of *uvsD* were highly sensitive to low concentrations of HU, whereas other genes displayed no or only limited sensitivity (Figure 1). All alleles of *uvsB* and *uvsD* displayed similar sensitivity to HU, consistent with the previous finding that they belong to the same complementation group (Kafer and Mayor, 1986). Interestingly, *uvsB* and *uvsD* mutant strains displayed similar sensitivity to HU as a strain (*nimX*^{cdc2AF}), which contains a single copy of NIMX^{cdc2} that is nonphosphorylatable on the inhibitory tyrosine and threonine residues (Figure 1). In addition, a strain in which the major NIMX^{cdc2} tyrosine kinase ANKA^{wee1} has been deleted (Δ*ankA*^{wee1}) was less sensitive to HU than the *uvsB*, *uvsD* and *nimX*^{cdc2AF} mutant strains (Figure 1). These results are consistent with *uvsB* and *uvsD* playing roles in checkpoint regulation in response to both DNA damage and prolonged S-phase and suggest that they may function in the pathway leading to tyrosine phosphorylation of NIMX^{cdc2}.

uvsB and uvsD Mutants Are Defective in the G2 DNA Damage and Prolonged S Phase Checkpoints over Mitosis

To confirm that the sensitivity of *uvsB* and *uvsD* mutants to DNA damage was due to loss of checkpoint control over entry into mitosis, we used strains that also carried the *nimA5* temperature-sensitive mutation. Cells arrested in G2 at the *nimA5* arrest point were either treated or not treated with 0.02% MMS to elicit DNA damage. Cells were then released in the absence of MMS to the permissive tempera-

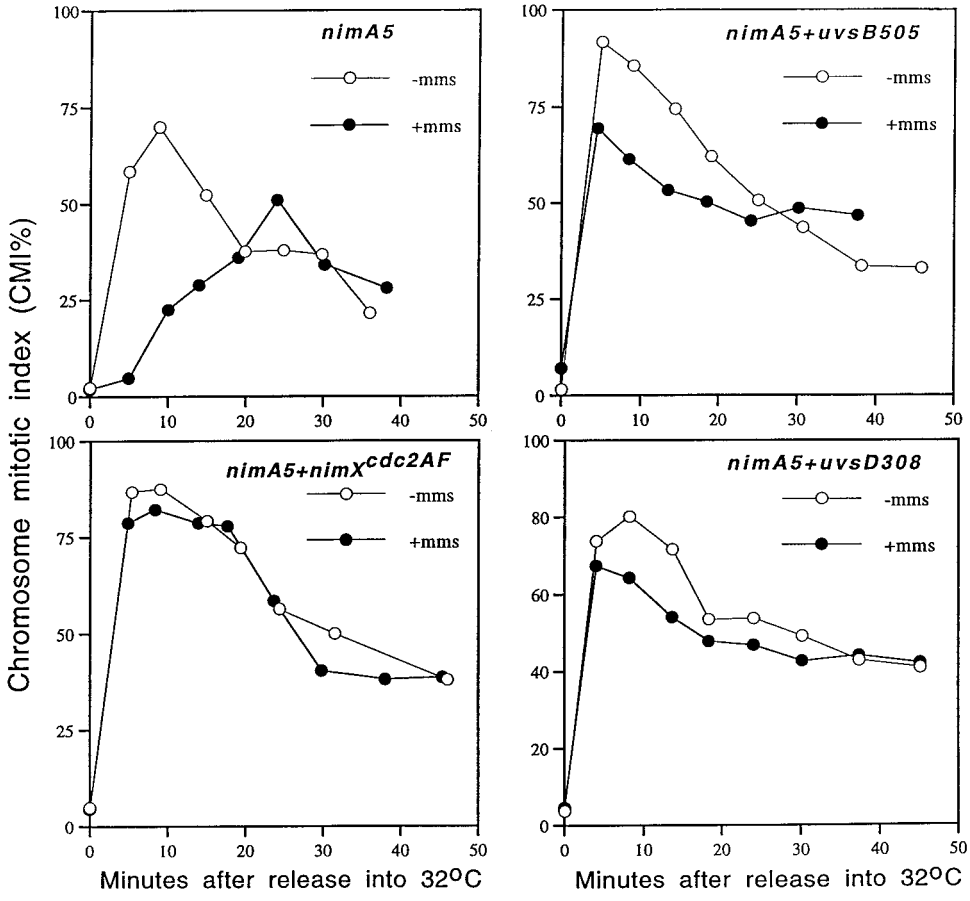

Figure 2. *uvsB* and *uvsD* mutants fail to arrest in response to DNA damage in G2. The four indicated *nimA5* strains were arrested in G2 at 42°C before downshift to 32°C to allow synchronous release into mitosis. Cells were released with or without MMS-induced DNA damage as indicated, and the chromosome mitotic index was determined in >200 cells per time point after staining DNA of fixed cells with DAPI.

ture and entry into mitosis followed by determining the chromosome mitotic index at time points after release. Under these conditions, entry into mitosis is delayed 15 min in the presence of DNA damage in the *nimA5* mutant (Figure 2). In contrast, *uvsB505* + *nimA5* and *uvsD308* + *nimA5* double mutants failed to arrest in response to G2 DNA damage and entered mitosis with similar kinetics as when MMS was not included and like the *nimX*^{cdc2AF} + *nimA5* double mutant in the presence of MMS (Figure 2). These data suggest that the sensitivity to DNA damage of *uvsB* and *uvsD* mutants is due to premature entry into mitosis with damaged DNA.

We next investigated whether the sensitivity of *uvsB* and *uvsD* mutants to low concentrations of HU was due to premature entry into mitosis using a wild-type and a *nimX*^{cdc2AF} mutant strain as controls. Conidiospores were germinated in the presence or absence of 6 mM HU, and the chromosome mitotic index was determined at time points after germination. Consistent with previous studies, entry into mitosis was markedly delayed in the wild-type strain in the presence of HU, but the *nimX*^{cdc2AF} mutant strain, which cannot be negatively regulated by tyrosine phosphorylation, entered mitosis 1 h earlier than the wild-type strain in the presence of HU (Ye *et al.*, 1996; our unpublished results). Similarly, *uvsB* and *uvsD* mutants germinated in the presence of 6 mM HU entered mitosis 30 min earlier than the wild-type strain germinated under the same conditions. Thus, like mutations that impair tyrosine phosphorylation of NIMX^{cdc2}, mutations in *uvsB* and *uvsD* lead to sensitivity to low concentrations of HU, at least in part because of loss of the S-phase checkpoint over entry into mitosis. Supporting this, the lethality of *uvsB* and *uvsD* mutants germinated in the presence of 6 mM HU could be rescued if premature entry into mitosis was prevented by arresting cells in G2 at the *nimA5* arrest point followed by release to the permissive temperature in the absence of HU (our unpublished results).

uvsB and uvsD Also Have a Function That Is Independent of Tyrosine Phosphorylation of NIMX^{cdc2}

Although the *nimX*^{cdc2AF} mutant is sensitive to DNA damage elicited during the cell cycle, remarkably this strain is no more sensitive than a wild-type strain when quiescent conidiospores are subjected to UV irradiation (Ye *et al.*, 1997b). In contrast, previous studies have demonstrated that *uvsB* and *uvsD* mutant conidiospores are highly sensitive to DNA damage (Kafer and Mayor, 1986). To directly compare the sensitivity of these mutants to DNA damage, we determined their viability after UV irradiation, using the DNA damage repair-deficient *uvsH4*^{rad18} mutant (Kafer and Mayor, 1986; Yoon *et al.*, 1995) and wild-type strains as controls. *uvsB* and *uvsD* mutants were more sensitive to UV irradiation than the *nimX*^{cdc2AF} mutant when either conidiospores or germlings

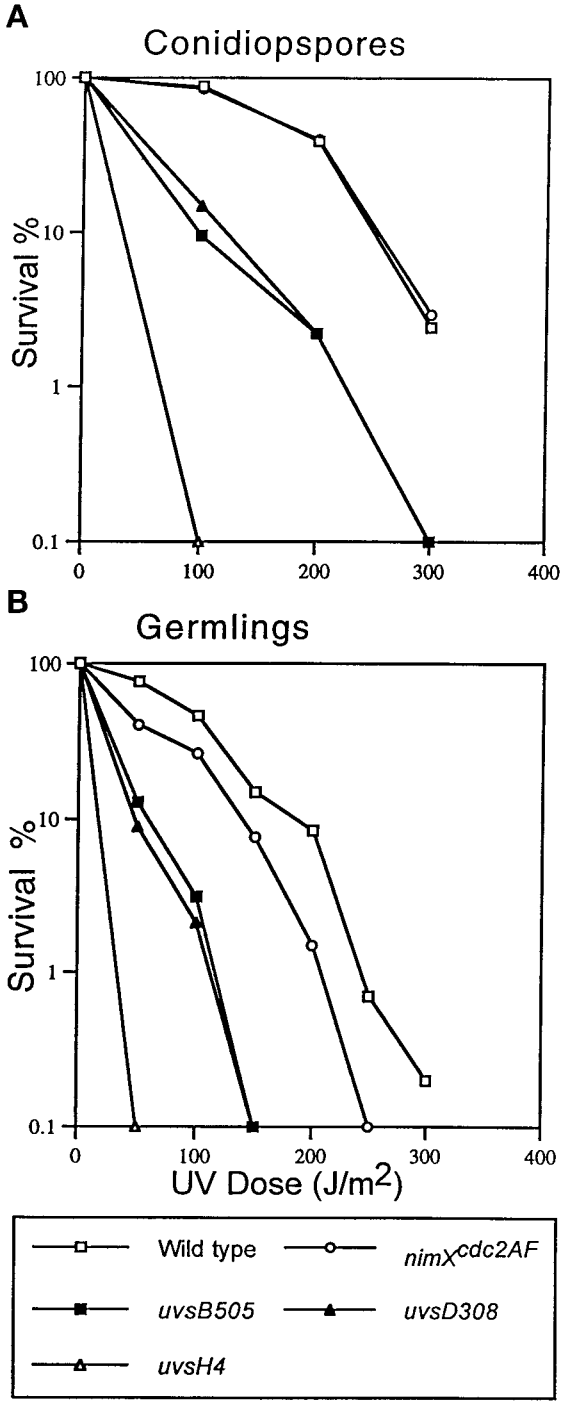

Figure 3. Differential UV sensitivity of strains deficient in the G2 DNA damage checkpoint. Conidiospores (250 per plate, two plates per strain) of wild-type and *nimX*^{cdc2AF}, *uvsB505*, *uvsD308*, and *uvsH4* mutant strains were spread on YAG plates and either immediately UV irradiated as indicated (A) or incubated at 32°C for 4.5 h to allow spore germination and then UV irradiated (B). After irradiation the plates were incubated at 32°C for 2 d to allow colony formation. The percent survival after DNA damage by UV irradiation is expressed as the percentage of colonies produced in the absence of treatment.

were irradiated (Figure 3, A and B). As shown previously (Ye *et al.*, 1997a), *nimX*^{cdc2AF} mutant conidiospores displayed sensitivity to UV irradiation similar to that of the wild-type strain, but *uvsB505* and *uvsD308* mutant conidiospores displayed significant sensitivity to UV irradiation (Figure 3A). These data indicate that *uvsB* and *uvsD* have functions in response to DNA damage that are independent of tyrosine phosphorylation of NIMX^{cdc2}. In addition, *uvsB505* and *uvsD308* mutants are not as sensitive as the repair deficient *uvsH4*^{rad18} strain (Figure 3A), suggesting that these mutants are not completely DNA damage repair deficient.

uvsB Is a rad3 Homologue, and uvsD Displays Homology to rad26

We cloned *uvsB* and *uvsD* by complementation of the HU sensitivity of the *uvsB505* and *uvsD308* alleles using an *A. nidulans* plasmid-based genomic DNA library. Positive transformants were also fully complemented for sensitivity to MMS (Figure 4A). Single-copy integration was confirmed by Southern blotting, and plasmids were recovered from complemented strains (Osmani *et al.*, 1987). Recovered plasmids were retransformed into the original mutant strains, and complementing plasmids were sequenced. We confirmed homologous integration at the *uvsB* locus by two-step gene replacement (Osmani *et al.*, 1987). A database search revealed extensive homology of *uvsB* to the *S. pombe* checkpoint rad gene *rad3*; however, the genomic sequence failed to identify any large open reading frames. To obtain the coding sequence for *uvsB*, RACE-PCR was performed, and sequencing of the 5′ and 3′ RACE-PCR products identified a single large open reading frame of 7365 bp coding for a 2454-amino-acid protein assuming that the first in-frame methione is used for translational initiation. Comparison of the gDNA and the cDNA identified a coding region of 8768 bp containing 25 introns, which are present throughout the coding region (GenBank accession number AF178850). This is a remarkably high number of introns given that the *S. pombe rad3* gDNA sequence consists of a single open reading frame (Bentley *et al.*, 1996). Sequence comparison confirmed that *uvsB* is a *rad3* homologue with 34% overall identity (Figure 4B) and indicated that it also displays high homology to other members of this family, including the human *ATM* (22%) and *ATR* (28%) genes. UVSB is most homologous to rad3 in its kinase domain, where it displays a higher level of homology to *rad3* (58% identity) than does the next closest *rad3* homologue, ATR (53% identity; Figure 4B) (Bentley *et al.*, 1996).

Sequencing of *uvsD* gDNA and RACE-PCR products identified an open reading frame of 2377 bp containing a single intron and coding for a 778-amino-acid protein (GenBank accession number AF180367). We confirmed integration at the *uvsD* locus by sequencing the mutant allele in the complemented strain, as we were unable to perform a two-step gene replacement because the complemented strain failed to undergo a self-cross. This identified a single point mutation substituting a stop codon instead of the glutamine at codon number 237 (CAG→TAG). Database searches identified UVSD as having highest homology to rad26 (Figure 4C). UVSD and rad26 both contain a coiled coil domain followed by a putative nuclear localization sequence (Figure 4C). Given the similar phenotypes of *uvsD* and *rad26* mutants and the sequence and structural homologies of these

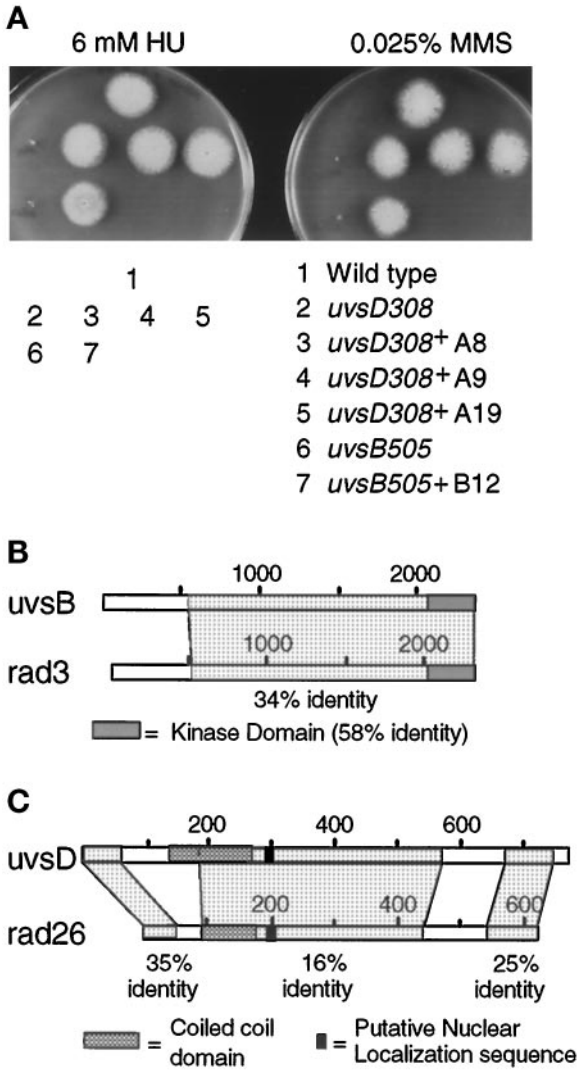

Figure 4. Identification of *uvsB* as a *rad3* homologue and *uvsD* as a *rad26*-like gene. (A) Growth of the wild-type and the *uvsB505* and *uvsD308* mutant strains and the complemented transformants *uvsD308*$^+$ A8, A9, A19, and *uvsB505*$^+$ B12, as indicated 3 d after inoculation at 32°C. (B) Schematic diagram showing the kinase domain of UVSB and the region of homology to rad3. (C) Schematic diagram of UVSD showing regions of homology with rad26, including a coiled coil domain followed by a putative nuclear localization sequence as indicated.

genes, *uvsD* is likely to be the first identified *rad26* homologue.

rad3 and *rad26* are nonessential genes in *S. pombe* (Jimenez *et al.*, 1992; Al-Khodairy *et al.*, 1994); however, the *S. cerevisiae rad3* homologue *MEC1* plays an essential role in budding yeast (Kato and Ogawa, 1994). To determine whether *uvsB* and *uvsD* have essential functions in *A. nidulans*, we performed targeted gene disruption of the respective genes (see MATERIALS AND METHODS; Osmani *et al.*, 1994). Disruptions were confirmed by Southern blotting, and strains were analyzed for sensitivity to HU and MMS (our unpublished results). The resulting Δ*uvsB* and Δ*uvsD* strains were viable and displayed sensitivity to HU and MMS similar to that of the respective mutant alleles (our unpublished results). Thus, similar to *S. pombe rad3* and *rad26*, *uvsB* and *uvsD* are apparently nonessential genes that are involved in checkpoint regulation over G2 DNA damage and prolonged S-phase.

Deregulation of cdc2 Kinase Activity during Prolonged S Phase Leads to an Abnormal Mitosis and Subsequent Over-Replication of DNA

Phenotypically, *uvsB*^{rad3} and *uvsD*^{rad26} mutants are similar to strains that are unable to tyrosine phosphorylate NIMX^{cdc2}. To investigate the phenotype of premature entry into mitosis, we germinated the Δ*ankA*^{wee1} strain in the presence of 6 mM HU and examined germlings by DAPI staining to visualize DNA (Figure 5, B–D). Under these conditions, wild-type strains delay entry into mitosis (Ye *et al.*, 1996), but nuclear division and migration occur normally (Figure 5A). In contrast, the Δ*ankA*^{wee1} germlings displayed striking, abnormal DNA morphologies consisting of polyploid nuclei, which became highly stretched as hyphal growth continued (Figure 5, B and C). Failure to segregate DNA is expected for cells prematurely entering mitosis from S-phase; however, the over-replication of DNA was completely unexpected. To confirm that cells displaying over-replicated DNA were in interphase and not undergoing mitosis, we examined the microtubule network by immunofluorescent staining at 1-h intervals after germination of the Δ*ankA*^{wee1} strain in 6 mM HU. Cells with polyploid nuclei always displayed interphase patterns of microtubule staining (Figure 5D), and mitotic spindles were never observed after the initial premature mitosis, even though these cells continued to replicate their DNA, resulting in the formation of polyploid nuclei. The *nimX*^{cdc2AF} mutant strain is more sensitive to low concentrations of HU than the Δ*ankA*^{wee1} strain (Ye *et al.*, 1996). Examination of germlings of a *nimX*^{cdc2AF} strain germinated in the presence of 6 mM HU revealed that in marked contrast to the wild-type strain (Figure 5A), these cells displayed massive nuclei, which were often highly stretched in both germlings (Figure 5E) and hyphae (Figure 5F). The nuclei of the Δ*ankA*^{wee1} and *nimX*^{cdc2AF} strains germinated in low concentrations of HU are clearly polyploid, suggesting that over-replication of DNA is occurring in these cells in the absence of mitosis. This can clearly be seen in Figure 5F, in which the DNA of one nucleus has been extensively stretched into the three separate branched hyphae. Because cells over-replicating their DNA do not undergo mitosis, this dramatic stretching cannot be due to mitotic forces but rather may be the result of the action of *nud* genes, which function to position individual nuclei within the cytoplasm (Morris *et al.*, 1998). We therefore induced rereplication in a *nudC3* + *nimX*^{cdc2AF} double mutant at the restrictive temperature for *nudC3.* Although the rereplication phenotype was still observed, no stretching of nuclei occurred (our unpublished results). This demonstrates that the nuclear stretching is the result of attempted migration of a single nucleus. In extreme examples (Figure 5F), large polyploid nuclei were found stretched into several different hyphal branches. Clearly such multidirectional stretching cannot be the result of abortive mitosis.

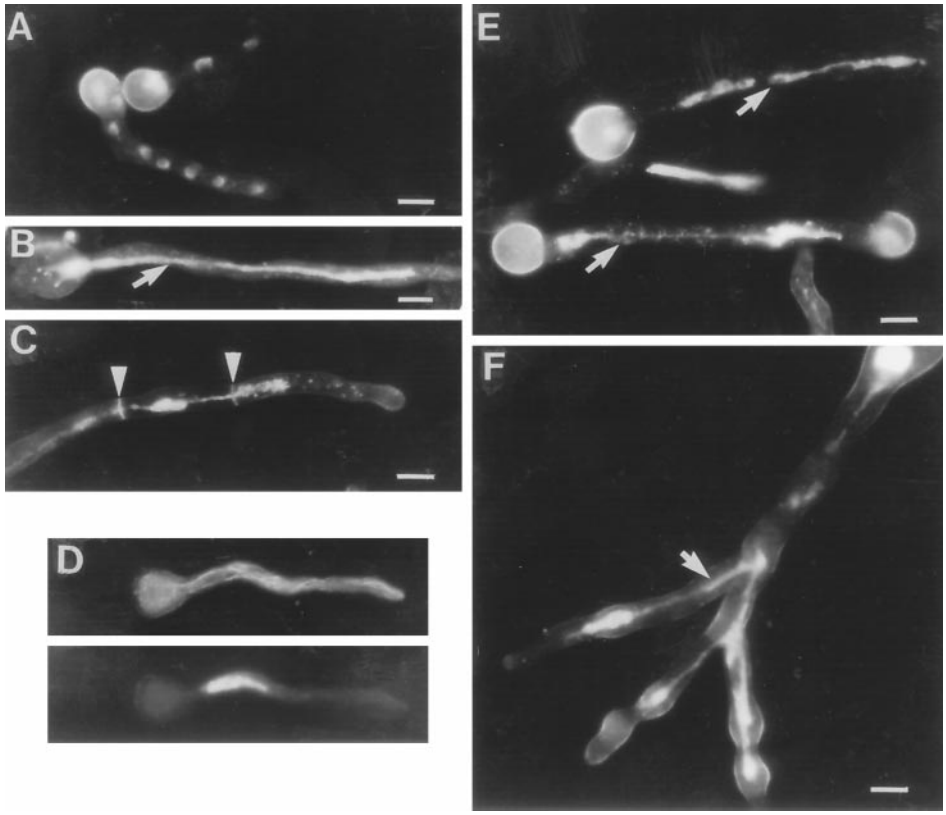

Figure 5. DNA rereplication in strains that are unable to tyrosine phosphorylate NIMX^{cdc2} in response to a slowed S-phase. (A–C) DAPI staining for DNA of wild-type cells 11 h after germination in 6 mM HU (A), a Δ*ankA*^{wee1} germling 11 h after germination in the presence of 6 mM HU (B), and a Δ*ankA*^{wee1} germling 15 h after germination. Arrowheads indicate septa stained by Calcofluor. (D) Tubulin staining of a Δ*ankA*^{wee1} germling 10 h after germination in 6 mM HU. The top panel shows tubulin staining, and the lower panel shows DAPI staining of DNA in the same germling. (E) DAPI staining of *nimX*^{cdc2AF} mutant germlings grown in 6 mM HU for 11 h. (F) Hyphae after growth of a *nimX*^{cdc2AF} strain for 14 h in 6 mM HU. Arrows indicate stretched DNA (B, D, and F). Bars, 5 μm.

Septum formation was often deregulated in mutants displaying the rereplication phenotype and was observed more frequently and in shorter germlings compared with wild-type strains germinated under the same conditions (Figure 5C; our unpublished results). Moreover, septation often occurred in the absence of nuclear division in these mutants, resulting in a cut-like phenotype (Figure 5C).

To further examine the morphology of the nuclei in cells displaying the rereplication phenotype, germlings of the Δ*ankA*^{wee1} strain were grown for 10 h in the absence or presence of 6 mM HU and subjected to electron microscopy. In the absence of HU nuclear division occurred normally, with four distinct nuclei being apparent in the cell shown in Figure 6A. In striking contrast, cells grown in HU displayed abnormal, giant nuclei, which were polyploid (Figure 6B), demonstrating that DNA replication had continued in the absence of an effective mitosis, and that the DNA is maintained in a single nuclear membrane.

In fission yeast *rad3* and *rad26* are thought to function in checkpoint control through a pathway that regulates tyrosine phosphorylation of cdc2 (Al-Khodairy and Carr, 1992; Furnari *et al.*, 1997; Uchiyama *et al.*, 1997; Lindsay *et al.*, 1998; Martinho *et al.*, 1998). Given that strains unable to tyrosine phosphorylate NIMX^{cdc2} entered mitosis early in the presence of low concentrations of HU and subsequently over-replicated their DNA, we were interested in determining whether the same occurred in *uvsB*^{rad3} and *uvsD*^{rad26} mutants. We examined *uvsB505*^{rad3} and *uvsD308*^{rad26} germlings grown in the presence of 6 mM HU for 12 h by DAPI staining and observed similar over-replication phenotypes as in the Δ*ankA*^{wee1} and *nimX*^{cdc2AF} mutants under these conditions (our unpublished results; Figure 7).

To estimate the ploidy of cells that had undergone over-replication of DNA, we measured the relative nuclear fluorescence of nuclei in cells from wild-type, *nimX*^{cdc2AF}, *uvsB505*, and *uvsD308* strains germinated for 12 h in the presence of 6 mM HU (Figure 7). A time point of 12 h was chosen for this experiment, even though over-replication continued to occur, because DNA subsequently became highly stretched, making measurements difficult. Even at 12 h, the nuclei of *nimX*^{cdc2AF}, *uvsB505*, and *uvsD308* germlings were clearly polyploid displaying 4.8, 3.9, and 5.2 times, respectively, the DNA content of wild-type nuclei when grown in the presence of 6 mM HU (Figure 7).

The rereplication phenotype described above may be a consequence of entry into mitosis before the completion of DNA replication, which would subsequently cause defects in DNA segregation as observed in Figure 5. To determine whether cells displaying over-replicated and incompletely segregated DNA had undergone an abnormal mitosis, we germinated the Δ*ankA*^{wee1} mutant in 6 mM HU for 6.5 h and determined the average spindle length of cells undergoing the first mitosis. In comparison with an average spindle length of 2.3 μm for normal wild-type cells undergoing their first mitosis, the mean spindle length of Δ*ankA*^{wee1} germlings grown in 6 mM HU was only 1.4 μm. This was largely because of the failure of the Δ*ankA*^{wee1} cells to elongate their spindles in the presence of HU with no spindles >4 μm being observed in this strain compared with the wild-type strain, in which 17% of spindles were >4 μm long. Together,

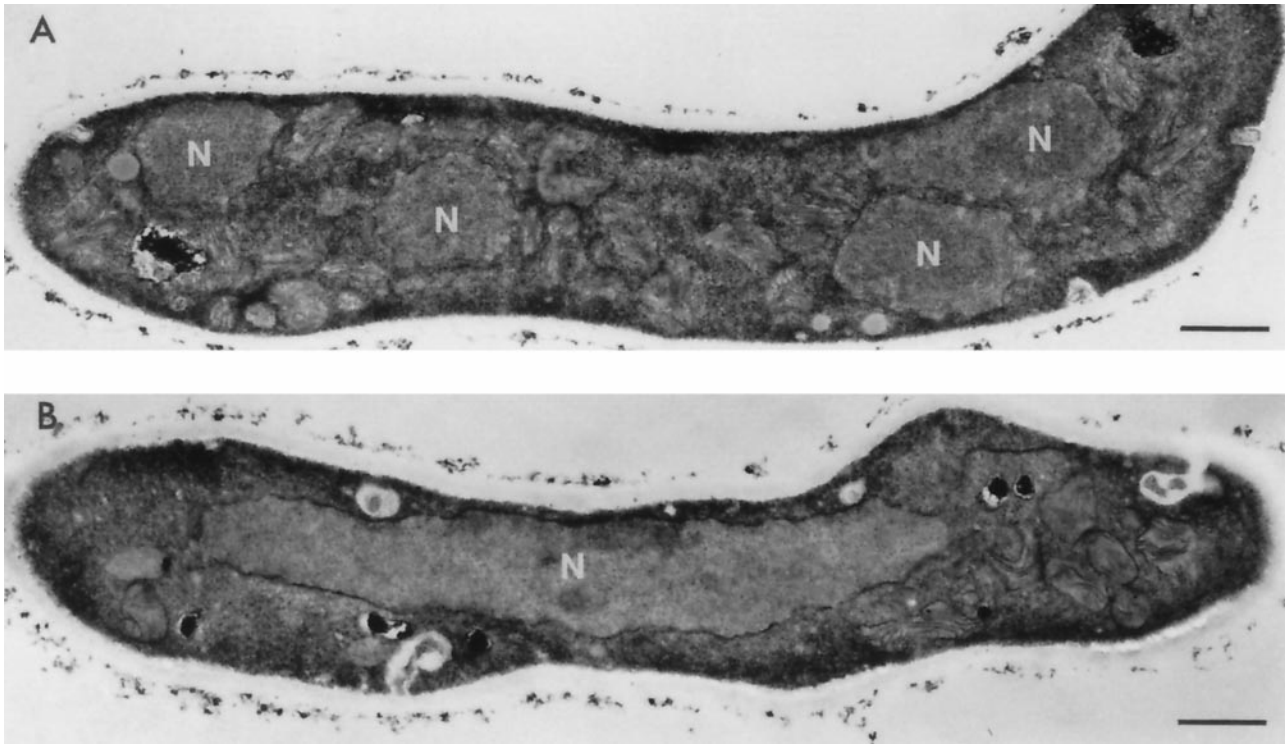

Figure 6. Electron micrographs of Δ*ankA*^{wee1} control and over-replicating germlings. (A) Germling germinated in the absence of HU for 10 h. (B) Germling germinated in the presence of 6 mM HU for 10 h displaying a massive polyploid nucleus. N, nuclei. Bar, 1 μm.

these data strongly suggest that cells entering mitosis before the completion of DNA replication undergo an abnormal mitosis leading to the failure of DNA segregation and the subsequent over-replication of DNA.

Combination of Impaired APC Function and the nimX^{cdc2AF} Mutation Results in the Rereplication Phenotype in the Absence of HU

The APC1 homologue BIME has been previously demonstrated to play a role in S-phase checkpoint regulation (Ye *et al.*, 1996, 1997b). Specifically, the *bimE7*^{APC1} mutation can override the S-phase arrest induced by 100 mM HU in a *nimX*^{cdc2AF} mutant by a mechanism that leads to the activation of the NIMA kinase. Similarly, the *bimE7*^{APC1} mutation also negates S-phase arrest induced by inactivation of the mini chromosome maintenance protein *nimQ*^{mcm2} at the restrictive temperature (James *et al.*, 1995; Ye *et al.*, 1997b). In both of these cases, it is a combination of compromised APC function and lack of cdc2 tyrosine phosphorylation that overrides the S-phase arrest (Ye *et al.*, 1996, 1997b). The ability of *bimE7*^{APC1} to allow entry into mitosis of a *nimX*^{cdc2AF} mutant arrested in S-phase by 100 mM HU at 32°C (the permissive temperature for *bimE7*^{APC1}) suggests that the mutant BIME^{APC1} is not completely functional in this mutant at 32°C. We have previously observed that the *nimX*^{cdc2AF} + *bimE7*^{APC1} double mutant is synthetically lethal and grows poorly at 32°C. This is likely to be a consequence of the unregulated entry of cells into mitosis during S-phase, which is analogous to what occurs in *uvsB*, *uvsD* and *nimX*^{cdc2AF} mutants grown in the presence of low concentrations of HU. With this in mind, we examined the phenotype of the *nimX*^{cdc2AF} + *bimE7*^{APC1} double mutant germinated at 32°C by DAPI staining. These cells either contained nuclei that displayed incomplete DNA segregation (Figure 8, A and B), or polyploid nuclei (Figure 8C). These phenotypes are almost identical to those of *uvsB*, *uvsD* and *nimX*^{cdc2AF} mutants grown in the presence of low concentrations of HU. These data suggest that the *bimE7*^{APC1} mutation in combination with lack of tyrosine phosphorylation of NIMX^{cdc2} can promote premature entry into mitosis at varying stages of S-phase and that rereplication seen in previous experiments is not a consequence of the presence of HU. Interestingly, if the *nimX*^{cdc2AF} + *bimE7*^{APC1} double mutant strain was germinated in low concentrations of HU at the restrictive temperature for *bimE7*^{APC1}, cells arrested with a BIM phenotype (Figure 8D) instead of the rereplication phenotype observed in the *nimX*^{cdc2AF} mutant under these conditions. Thus, completely inhibiting APC function prevents rereplication of DNA by arresting cells in mitosis.

Mitotic Kinase Activities Are Decreased in Cells Undergoing DNA Rereplication

The rereplication phenotype appears to be a consequence of over-replication of DNA after premature entry into mitosis. In *S. pombe,* cells deleted for *cdc13*^{cyclinB} (Hayles *et al.*, 1994) or overexpressing the cdc2/cdc13$^{cyclin\ B}$ kinase inhibitor *rum1* (Moreno and Nurse, 1994), undergo multiple rounds of replication without mitosis, suggesting that rereplication of

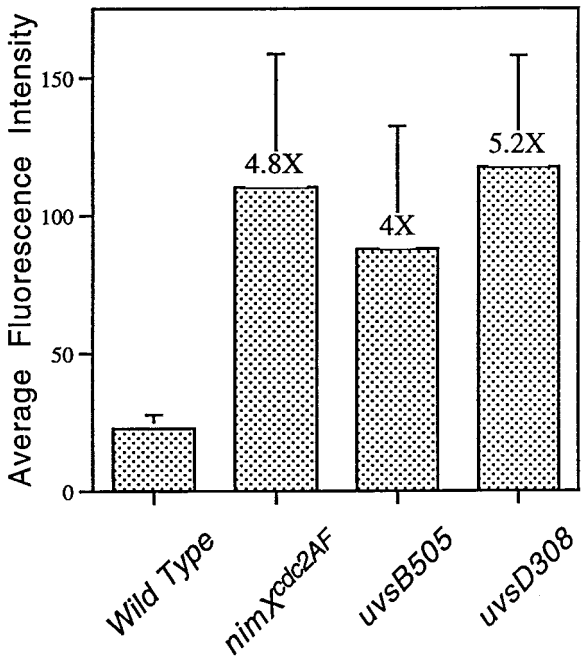

Figure 7. Comparison of the relative DNA content of cells undergoing DNA replication. Average fluorescence intensity of nuclei in 12 cells (6 cells from two independent experiments) of the indicated strains germinated in the presence of 6 mM HU for 12 h. Error bars represent the SD observed for each strain.

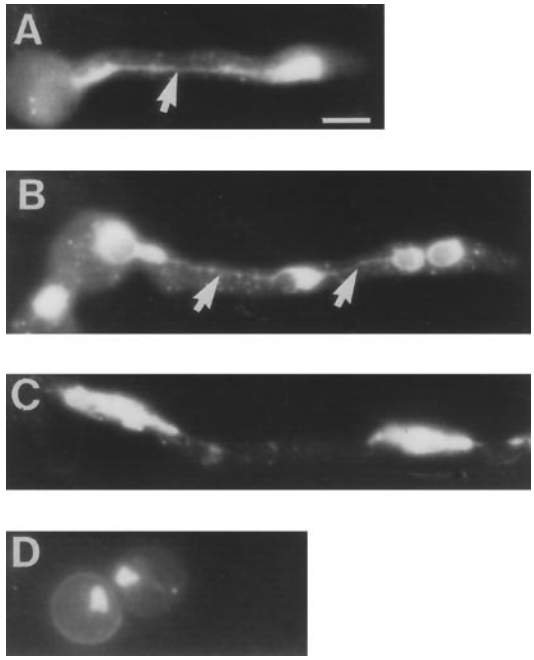

Figure 8. *nimX*^{cdc2AF} + *bimE7*^{APC1} double mutant cells display polyploid nuclei. DAPI staining of cells showing representative nuclear morphologies observed in the double mutant germlings are shown. (A) 12-h-old germling with a highly stretched large nucleus (17% of cells). (B) Germling showing incomplete DNA segregation indicated by the arrows (50% of cells). (C) Part of a hyphae showing large abnormal nuclei (33% of cells). (D) Double mutant spores 12 h after germination at 42°C, showing the typical mitotic block phenotype of the *bimE7* mutation with highly condensed DNA. Arrows indicate stretched nuclei. Bar, 5 μm.

DNA may be induced by the failure to accumulate cdc2 kinase activity during S-phase. In *A. nidulans*, the kinase activities of both NIMX^{cdc2} and NIMA are required for entry into mitosis (Osmani *et al.*, 1991a; Ye and Osmani 1997). To examine these kinase activities in cells undergoing rereplication, we performed kinase assays on extracts prepared from log phase cultures of a wild-type and a *nimX*^{cdc2AF} strain at various time points after addition of 10 mM HU (Figure 9). Under these conditions, the rereplication phenotype is evident in the *nimX*^{cdc2AF} strain after the first premature mitosis, and large polyploid nuclei are clearly evident by 10 h. As expected, NIMX^{cdc2} kinase activity was considerably higher compared with wild-type cells before the addition of HU, because negative regulation of this kinase by tyrosine phosphorylation cannot occur in this mutant (Figure 9A). However, NIMX^{cdc2} kinase activity decreased markedly by 6 h after addition of HU in the *nimX*^{cdc2AF} strain (Figure 9A). NIMA kinase levels also decreased during this period, consistent with NIMA being activated downstream of NIMX^{cdc2} (Figure 9B, Ye *et al.*, 1995). This decrease in NIMX^{cdc2} kinase activity was not due to a decrease in levels of NIMX^{cdc2} protein but rather the decrease in levels of its cyclin partner NIME^{cyclinB} (Figure 9C). In contrast, the activity of the mitotic kinases fluctuated after addition of HU to wild-type cells, which undergo a normal, albeit delayed, mitosis under these conditions (Figure 9, A and B). These data indicate that over-replication of DNA occurs in the presence of low NIMX^{cdc2} kinase activity in the *nimX*^{cdc2AF} strain after premature mitotic entry in the presence of incomplete DNA replication.

DNA Over-Replication Continues in the Presence of High NIMX^{cdc2} Kinase Activity

Given that DNA over-replication can be induced in *S. pombe* if cyclin B/cdc2 kinase activity is eliminated (Hayles *et al.*, 1994; Moreno and Nurse, 1994), we next determined whether maintaining a high level of NIMX^{cdc2} kinase activity could prevent the rereplication phenotype we observe in the *nimX*^{cdc2AF} mutant strain. To do this, we used a *nimX*^{cdc2AF} strain that also contained a nondegradable form of NIME$^{cyclin\ B}$ under the control of the inducible *alcA* promoter. Cells grown on coverslips in minimal acetate medium (repressing) were allowed to undergo the first premature mitosis in the presence of 6 mM HU, after which expression of nondegradable of NIME^{cyclinB} was allowed in glycerol medium. Under these conditions, cells still clearly displayed the rereplication phenotype characterized by large polyploid nuclei (Figure 10B). To follow NIMX^{cdc2} kinase activity biochemically after induction of nondegradable NIME$^{cyclin\ B}$, cells were grown in liquid culture to an early log phase before the addition of 6 mM HU for 4 h to cause premature mitosis. DAPI staining of aliquots of these HU-treated cells indicated that they did not display the rereplication phenotype at this time (our unpublished results). Expression of nondegradable NIME$^{cyclin\ B}$ was then allowed by medium change, and samples were analyzed at the indicated times for NIMX^{cdc2} kinase activity (Figure

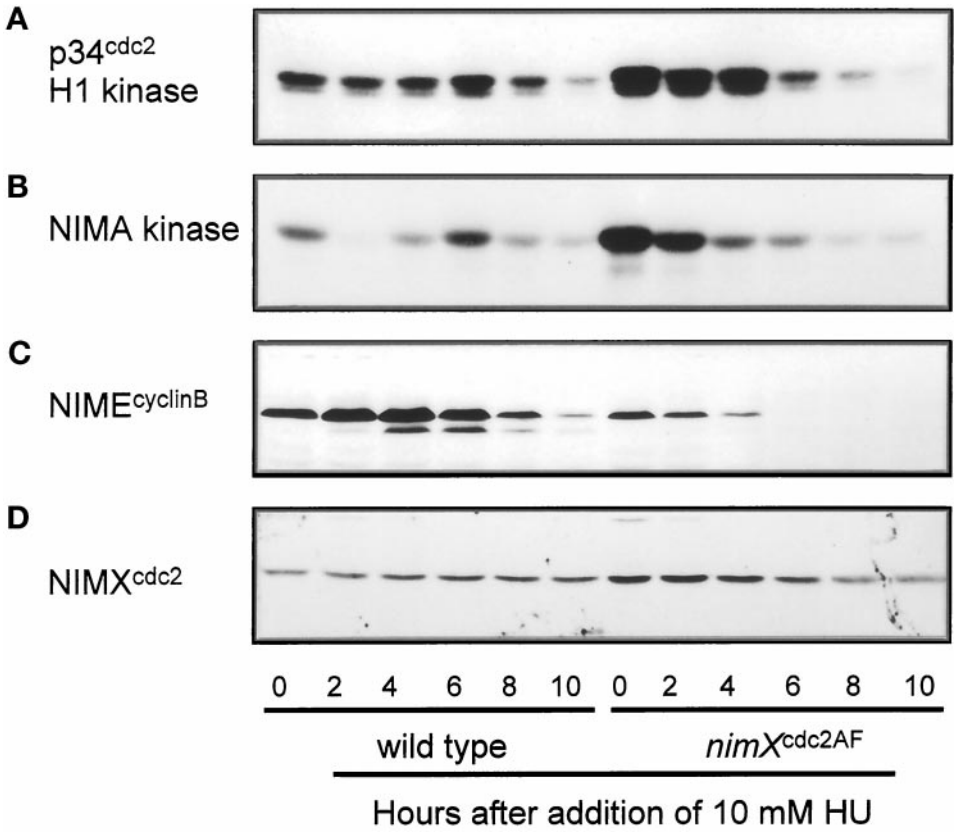

Figure 9. Loss of NIMX^{cdc2} and NIMA kinase activities and proteolysis of NIME^{cyclinB} in *nimX^{cdc2AF}* mutant cells after addition of 6 mM HU. HU was added to log phase cultures of either a wild-type or the *nimX^{cdc2AF}* mutant strain to the final concentration of 10 mM, and samples were collected at the time intervals indicated. NIMX^{cdc2} (A) and NIMA (B) kinase activities were assayed using histone H1 or β-caesin as substrate. NIME$^{cyclin\ B}$ (C) and NIMX^{cdc2} (D) protein levels were determined by Western blotting.

10C) and by DAPI staining. These cells still displayed the rereplication phenotype (our unpublished results), even though NIMX^{cdc2} kinase activity was maintained at a high level (Figure 10C). This indicates that loss of NIMX^{cdc2} kinase activity is not essential for continuing over-replication of DNA. Together with the experiments described earlier, this suggests that the rereplication phenotype occurs as a result of loss of checkpoint regulation over entry into mitosis because of the inability to tyrosine phosphorylate NIMX^{cdc2}, but that the subsequent over-replication of DNA occurs independently of NIMX^{cdc2} activity.

DISCUSSION

Here we demonstrate that *A. nidulans uvsB* and *uvsD* play roles in checkpoint regulation over entry into mitosis in response to slow S-phase and G2 DNA damage. These genes display homology to two members of the checkpoint rad gene family of *S. pombe*, *rad3* and *rad26*, respectively. *rad3* and *rad26* are thought to function in checkpoint control through a pathway that regulates cdc2 tyrosine phosphorylation (Al-Khodairy and Carr, 1992; Furnari *et al.*, 1997; Uchiyama *et al.*, 1997; Lindsay *et al.*, 1998; Martinho *et al.*, 1998). Homology between *uvsD* and *rad26* exists throughout the coding region of the gene and includes a coiled coil domain followed by a putative nuclear localization sequence. Given these sequence and structural homologies and the similar phenotypes caused by mutations of these genes, *uvsD* is likely to be the first identified *rad26* homologue. *uvsB* is a member of the *rad3/ATR/ATM/MEC1/TEL1* family of proteins displaying highest homology to *rad3* in *S. pombe*. The human *ATM* (ataxia–telangiectasia mutated) gene is involved in G2 checkpoint control and when mutated leads to cancer predisposition (Savitsky *et al.*, 1995), emphasizing the importance of cell cycle checkpoint control and normal health in humans.

Over-replication of DNA is a consequence of the loss of coordination between S-phase and mitosis. Maintenance of an ordered progression of S-phase and mitosis is thought to be carried out by a replication-licensing system in which a replication-licensing factor binds chromatin early in the cell cycle, is removed from chromatin as DNA replicates, and is unable to rebind replicated chromatin until after the following mitosis (Blow, 1993; Chong *et al.*, 1995). Here we demonstrate that in *A. nidulans*, the initiation of mitosis before the completion of DNA replication results in an abnormal mitosis, after which cells subsequently undergo over-replication of DNA without ever attempting mitosis again. Quantitation of the average nuclear fluorescence intensity indicated that the nuclei of cells displaying the rereplication phenotype contained greater than four times the DNA of wild-type nuclei by 12 h germination in 6 mM HU. That over-replication occurs without cells attempting another mitosis is supported by the absence of mitotic spindles in all cells displaying polyploid nuclei. The rereplication phenotype was demonstrated in *uvsB* and *uvsD* mutant strains as well as in strains that were unable to tyrosine phosphorylate

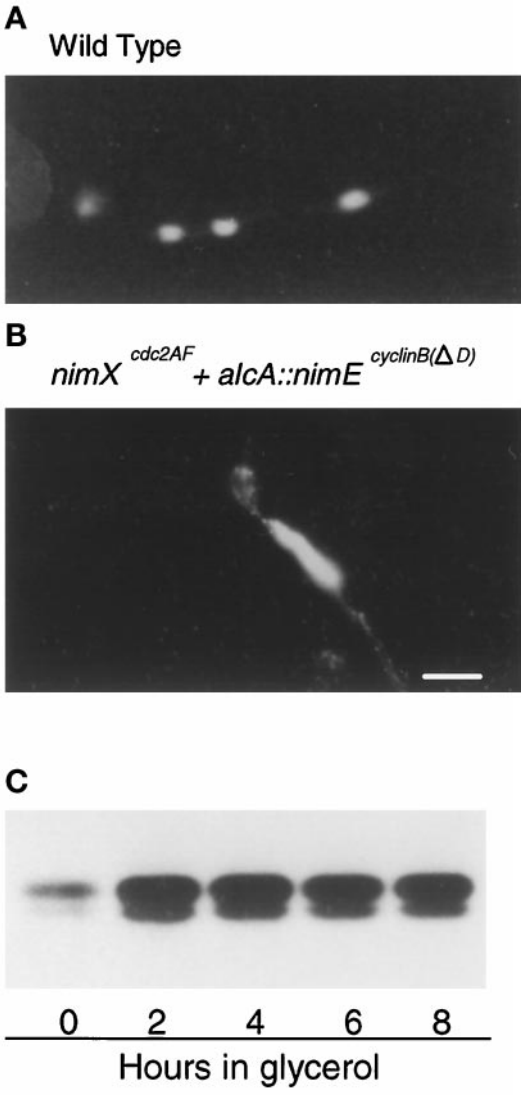

Figure 10. Maintaining high levels of NIMX^{cdc2} kinase activity in the *nimX*^{cdc2AF} mutant fails to prevent DNA over-replication. The *nimX*^{cdc2AF} + *alcA::nimE*$^{cyclin\ B\Delta D}$ strain, which contains a nondegradable (D box minus) *nimE*$^{cyclin\ B}$ under control of the *alcA* promoter, and a wild-type strain were first germinated in minimal medium containing acetate (repressing) in the presence of 6 mM HU. Cells were allowed to undergo the first premature mitosis, which occurs after 9 h in minimal medium. Expression of *nimE*$^{cyclin\ B\Delta D}$ was then allowed by transferring cells to glycerol-containing medium. DAPI staining of representative cells of (A) the wild-type and (B) *nimX*^{cdc2AF} + *alcA::nimE*$^{cyclin\ B\Delta D}$ strains after 7 h growth in glycerol-containing medium. Please note that when grown in minimal medium *A. nidulans* cells are much thinner than those shown in nutrient-rich medium (Figure 5). (C) p34^{cdc2} H1 kinase activity was isolated from the *nimX*^{cdc2AF} + *alcA::nimE*$^{cyclin\ B\Delta D}$ strain at the indicated times after transfer to glycerol-containing medium. Bar, 5 μm.

NIMX^{cdc2}. It is therefore likely that any breakdown in checkpoint regulation that allows cells prematurely into a defective mitosis will subsequently break the regulation that normally maintains ploidy in *A. nidulans*.

Although over-replication of DNA does not occur as a result of entry into mitosis before the completion of DNA replication or G2 DNA repair in *S. pombe*, over-replication can be induced under certain circumstances in this organism. Mutations in *S. pombe cut1* and *cut2* lead to a failure of sister chromatid separation, and when coupled with the *cdc11* mutation to prevent cytokinesis, cells over-replicate their DNA, resulting in the formation of polyploid nuclei (Creanor and Mitchison, 1990; Uzawa *et al.*, 1990; Funabiki *et al.*, 1996). In *A. nidulans*, mutations in the *cut1* homologue *bimB* and in the kinesin-like protein *bimC* lead to the failure of mitosis and nuclear division, but cells still replicate their DNA and form polyploid nuclei (Enos and Morris 1990; May *et al.*, 1992). In contrast to the *nimX*^{cdc2AF}, *uvsB* and *uvsD* mutants, however, *bimB* and *bimC* mutants attempt multiple abortive mitosis after the failure of the initial mitosis (Enos and Morris 1990; May *et al.*, 1992). In mutants lacking *bimB* and *bimC* function, cells progress through the cell cycle, but their replicated DNA is not segregated because of mechanical defects during mitosis. Such cells may delay in a mitotic-like state but fail to segregate DNA, exit mitosis, and resume DNA synthesis. They then reenter mitosis, fail to segregate DNA, and enter another round of DNA replication. Such repeated rounds of the cell cycle subsequently generate large polyploid nuclei. In contrast, premature mitosis induced by lack of *uvsB* or *uvsD* or tyrosine phosphorylation of NIMX^{cdc2} prevents progression through the first premature mitosis, but cells never again attempt mitosis, presumably because of lack of mitotic kinase activities. Indeed, loss of cdc2 kinase activity has also been associated with over-replication in *S. pombe*. Notably, severely impairing cdc2 kinase activity by deletion of the *cdc13*^{cyclinB} gene (Hayles *et al.*, 1994) or by overexpression of the cdc2/cdc13^{cyclinB} kinase inhibitor *rum1* (Moreno and Nurse, 1994) leads to the formation of polyploid cells. Endoreplication is also known to occur naturally in certain plant, *Drosophila*, and mammalian cells (Grafi and Larkins; Williams and Jackson, 1982; Sauer *et al.*, 1995; Datta *et al.*, 1996; Weiss *et al.*, 1998). Interestingly, similar to *S. pombe*, endoreplication appears to be associated with a loss of cdc2 kinase activity in terminally differentiating megakaryocytes (Datta *et al.*, 1996) and in maize endosperm (Grafi and Larkins, 1995). This suggests that low cdc2 kinase activity may allow over-replication of DNA.

We have demonstrated that NIMX^{cdc2} kinase activity decreases as a result of NIME^{cyclinB} proteolysis in the *nimX*^{cdc2AF} mutant under conditions that induce over-replication of DNA. However, maintaining high kinase activity by expressing a nondegradable form of NIME^{cyclinB} after premature mitosis failed to abolish over-replication of DNA. Thus, over-replication can still occur in the presence of high NIMX^{cdc2} kinase activity if cells are first allowed to undergo a premature mitosis. Further understanding of the rereplication phenotype described here should provide information on how DNA replication and successful exit from mitosis are interlinked and how some cells normally break this relationship to naturally produce polyploid cells.

Over-replication can also be induced in *S. pombe* by overexpression of the replication initiator cdc18 (Nishitani and Nurse, 1997; Greenwood *et al.*, 1998). Interestingly, cells overexpressing a version of cdc18 in which consensus CDK phosphorylatable sites have been mutated to alanine continue to over-replicate their DNA, even in the presence of

high levels of cdc2 kinase activity (Jallepelli *et al*., 1997). Furthermore, phosphorylation of these consensus CDK sites promotes wild-type cdc18 ubiquitin-dependent degradation (Jallepelli *et al*., 1997). This suggests that over-replication in *S. pombe* induced by lack of cdc2 activity may be due to defects in the degradation of cdc18. In addition to cdc18, proteolysis of rum1 (Kominami and Toda, 1997) and *Xenopus* wee1 (Michael and Newport, 1998) are also thought to be involved in progression into mitosis from S-phase. Our observation of over-replication in the $bimE7^{APC1}$ + $nimX^{cdc2AF}$ double mutant further implicates APC/C function in checkpoint control over mitotic entry. The observed loss of cyclin B protein in the $nimX^{cdc2AF}$ mutant undergoing rereplication suggests that the APC/C is active in these cells. It will be of interest to determine whether an *A. nidulans* cdc18 homologue plays a role in the rereplication phenotype described in this study and to define the precise role of proteolysis in maintaining the temporal order of S-phase and mitosis.

Our data also indicate that $uvsB^{rad3}$ and $uvsD^{rad26}$ have another role in response to DNA damage, which is independent of $NIMX^{cdc2}$. Specifically, UV irradiation of quiescent conidiospores causes a marked loss of viability of *uvsB* and *uvsD* mutants, but $nimX^{cdc2AF}$ conidiospores are no more sensitive than wild type. Germinating conidiospores do not enter the cell cycle directly, because they need to break dormancy and get their metabolism up and running. This delay presumably gives cells time to repair damaged DNA. The high UV sensitivity of *uvsB* and *uvsD* mutant conidiospores suggests that they have roles in the DNA damage response other than the regulation of $NIMX^{cdc2}$ tyrosine phosphorylation. *rad3* and *rad26* are thought to function early in the DNA damage response and may also play roles in the initiation of DNA repair. Supporting this, the *S. cerevisiae* $uvsB^{rad3}$ homologue *MEC1* is involved in the transcriptional activation of genes involved in DNA repair (Huang *et al*., 1998).

In conclusion, we have identified *A. nidulans* $uvsB^{rad3}$ and $uvsD^{rad26}$ as *rad3* and *rad26* homologues, respectively. The finding that, like in *S. pombe*, $uvsD^{rad26}$ functions in both the G2 DNA damage and prolonged S-phase checkpoints suggests functional conservation of this checkpoint gene. The isolation of $uvsD^{rad26}$ as a potential homologue of *S. pombe rad26* may enable human *rad26*-like genes to be identified to determine whether this class of checkpoint function is conserved in the same manner as $uvsB^{rad3}$. We further show that in *A. nidulans*, loss of checkpoint control over mitotic initiation leads to a defective mitosis followed by over-replication of genomic DNA. This defines a new mechanism by which endoreplication of DNA can be triggered. Finally, the dramatic rereplication phenotype we have defined will enable us to screen easily for further mutations causing defective checkpoint control over mitosis.

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