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The *Aspergillus nidulans npkA* Gene Encodes a Cdc2-Related Kinase That Genetically Interacts With the UvsB^{ATR} Kinase

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ABSTRACT

The DNA damage response is a protective mechanism that ensures the maintenance of genomic integrity. We have used *Aspergillus nidulans* as a model system to characterize the DNA damage response caused by the antitopoisomerase I drug, camptothecin. We report the molecular characterization of a p34^{Cdc2}-related gene, *npkA*, from *A. nidulans*. The *npkA* gene is transcriptionally induced by camptothecin and other DNA-damaging agents, and its induction in the presence of camptothecin is dependent on the *uvsB*^{ATR} gene. There were no growth defects, changes in developmental patterns, increased sensitivity to DNA-damaging agents, or effects on septation or growth rate in the *A. nidulans npkA* deletion strain. However, the Δ*npkA* mutation can partially suppress HU sensitivity caused by the Δ*uvsB*^{ATR} and *uvsD153*^{ATRIP} checkpoint mutations. We demonstrated that the *A. nidulans uvsB*^{ATR} gene is involved in DNA replication and the intra-S-phase checkpoints and that the Δ*npkA* mutation can suppress its intra-S-phase checkpoint deficiency. There is a defect in both the intra-S-phase and DNA replication checkpoints due to the *npkA* inactivation when DNA replication is slowed at 6 mM HU. Our results suggest that the *npkA* gene plays a role in cell cycle progression during S-phase as well as in a DNA damage signal transduction pathway in *A. nidulans*.

CONTROL of cell cycle progression is dependent on serine/threonine kinases called cyclin-dependent kinases (Cdk's). Cdk's can be grouped into two types: those that have and those that do not have a known cyclin partner. Such partners have been identified for some, but not all, members of the Cdc2 family, and the role in the cell cycle of Cdk's lacking cyclin partners remains unclear (de Falco and Giordano 1998). Only some Cdk's have roles in regulating the cell cycle. Most of the other Cdk's (*e.g.*, CDK7-9) have been implicated in the regulation of transcription as protein kinases that phosphorylate the C-terminal domain of RNA polymerase II. However, a number of other Cdc2-related kinases (Crk) either do not need to bind a cyclin to be active or have no cyclin partner that has been identified. When an activating cyclin partner is subsequently identified, the protein is reclassified as a Cdk (Ko *et al.* 2001). The Crk family comprises a diverse set of proteins that have 42–55% identity with the kinase domain of Cdc2 (Meyerson *et al.* 1992). Individual Crk family members are often referred to by the one-letter code of the amino acid sequence in the region corresponding to the "PSTAIRE" α-helix of Cdc2 that interacts with the cyclin. These are denoted on the basis of their amino acid homology in the PSTAIRE region of Cdc2, such as PITSLRE (Brambilla and Draetta 1994), PCTAIRE (Meyerson *et al.* 1992), PITAIRE (Lapidot-Lipson *et al.* 1992), and PISSLRE (Brambilla and Draetta 1994; Graña *et al.* 1994).

The DNA damage response is a protective mechanism that ensures the maintenance of genomic integrity during cellular reproduction. DNA damage takes several general forms including single- and double-strand breaks, base damage, and DNA-protein crosslinks. If left unrepaired, DNA damage can result in cell cycle arrest, cell death, and, if repaired incorrectly, the loss of genetic information or the accumulation of mutations that lead to cancer in multicellular organisms. DNA replication, gene transcription, DNA repair, and cell cycle checkpoints must all interlink to promote cell survival following DNA damage (Levitt and Hickson 2002). The two main signal transduction pathways that respond to DNA damage are conserved across evolution: the ATM (mutated in ataxia telangiectasia) and the ATR (ATM-Rad3-related) pathways (Rotman and Shiloh 1998; Zhou and Elledge 2000; Abraham 2001; Yang *et al.* 2003). The ATM pathway responds to the presence of double-strand breaks (DSBs). The ATR pathway also responds to DSBs, but more slowly than ATM. In addition, the ATR pathway can respond to agents that interfere with the function of replication forks, such as hydroxyurea

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(HU), ultraviolet light (UV), and DNA-alkylating agents such as methyl methanesulfonate (MMS; Nyberg *et al.* 2002; Osborn *et al.* 2002). The ATM/ATR kinases phosphorylate and activate signal transduction pathways that ultimately interface with the Cdk/cyclin machinery (Abraham 2001).

The filamentous fungus *Aspergillus nidulans* has been used as a model genetic system for the study of cell cycle control and the DNA damage response (for reviews, see Aist and Morris 1999; Goldman *et al.* 2002; Goldman and Kafer 2004; Osmani and Mirabito 2004). Two protein kinases, the $NimX^{Cdc2}$ and NimA, are coordinately required to initiate mitosis in *A. nidulans* (Osmani and Ye 1996; Ye *et al.* 1996). As in other eukaryotic cells, the DNA damage checkpoint in *A. nidulans* functions via phosphorylation of the $NimX^{Cdc2}$ Y15 (Ye *et al.* 1997). Loss of such checkpoint control regulation over mitosis can also cause DNA rereplication after mitosis (De Souza *et al.* 1999). The Wee1 ortholog AnkA and the Cdc25 ortholog NimT regulate the Y15 phosphorylation of NimX (Osmani *et al.* 1991; Ye *et al.* 1997; Kraus and Harris 2001), although it is not clear how their activity and/or localization are influenced by DNA damage. The DNA damage checkpoint also regulates septation in *A. nidulans* by modulating the activity of $NimX^{Cdc2}$ and requiring functional AnkA (Harris and Kraus 1998; De Souza *et al.* 1999).

We have used *A. nidulans* as a model system to genetically characterize the cellular response to the antitopoisomerase I drug, camptothecin (CPT; Bruschi *et al.* 2001; Kress Fagundes *et al.* 2003; Semighini *et al.* 2003). The basic mechanism of action for CPT is well characterized (Froelich-Ammon and Osheroff 1995). Briefly, CPT generates replication-mediated DNA double-strand breaks, which in turn induce reversible or permanent cell cycle arrest in G_2-M transition. In this study, we report the molecular characterization of *A. nidulans npkA*, a $p34^{Cdc2}$-related gene, which is transcriptionally induced by CPT and other DNA-damaging agents. We examined the role of the *npkA* gene in the DNA damage response. While *npkA* inactivation alone does not result in a clear phenotype, Δ*npkA* can partially suppress HU sensitivity of Δ$uvsB^{ATR}$ and $uvsD153^{ATRIP}$ mutations. In addition, the *npkA* genetically interacts with $bimE^{APC1}$ and $ankA^{wee1}$ genes during S-phase checkpoints. These results strongly suggest that the *npkA* gene plays a role in the DNA damage checkpoint during S-phase in *A. nidulans*.

MATERIALS AND METHODS

Strains, media, and methods of UV treatment: *Escherichia coli* strain KS272 [F^- Δ*lacX74 galE galK thi rpsL* Δ*phoA* (PvuII)] was used for propagation of the recombination vector pKOBEG and *A. nidulans* cosmids. *E. coli* strains were propagated in Luria-Bertani (LB) medium (1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) when selection for zeocin (Invitrogen, San Diego) resistance was applied. L-Arabinose or D-glucose was added as indicated to modulate expression of genes under control of the pBAD promoter (Guzman *et al.* 1995).

A. nidulans strains used are described in Table 1. Media were of two basic types—(1) a simple yeast extract "complete" medium with three variants: YAG (2% glucose, 0.5% yeast extract, 2% agar, trace elements); YUU, medium supplemented with 1.2 g/liter each of uracil and uridine; and liquid YG medium of the same compositions (but without agar); and (2) a modified minimal medium of 1% glucose, original high-nitrate salts, trace elements, 2% agar, pH 6.5, or minimal medium without glucose (MC). Trace elements, vitamins, and nitrate salts are described by Kafer (1977; appendix available on request from the author). Standard genetic techniques for *A. nidulans* were used for all strain constructions (Kafer 1977).

For the UV light viability assays, conidiospores (dormant in a quiescent G_0 state) were suspended in 0.2% Tween-20 and plated out on YUU plates (~100 conidia/plate). The plates were then irradiated immediately with UV using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and incubated at 32° for 48 hr to determine UV sensitivity of nondividing cells. To determine UV survival of dividing cells, conidiospores on YUU plates were first allowed to germinate for 4.5 hr in a 32° incubator for colony formation. By this time the germinated spores had entered the cell cycle and were about to undergo the first mitosis. These germlings were UV irradiated on the plates and then similarly incubated at 32° for 48 hr. Viability was determined as the percentage of colonies on treated plates compared to untreated controls.

Methods of intra-S-phase and DNA replication checkpoints: For the intra-S-phase checkpoint, conidiospores were inoculated onto coverslips in YUU medium with 0, 6, or 100 mM of HU. After 5–7 hr of incubation at 30°, coverslips with adherent germlings were transferred to fixative solution (3.7% formaldehyde, 50 mM sodium phosphate buffer pH 7.0, 0.2% Triton X-100) for 30 min at room temperature. Then, they were briefly rinsed with PBS buffer (140 mM NaCl, 2 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, pH 7.4) and incubated for 5 min in a solution with 100 ng/ml of 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical) and 100 ng/ml of calcofluor (fluorescent brightener, Sigma Chemical). After incubation with the dyes, they were washed with PBS buffer for 5 min at room temperature and then rinsed in distilled water and mounted in Citifluor. The material was photographed using a Zeiss epifluorescence microscope. The number of nuclei was assessed by DAPI staining. Germlings that had two or more nuclei after the HU incubation were scored as germlings that disrupted the S-phase blockage.

For the DNA replication checkpoint, 1.0×10^8 conidia were inoculated in YUU medium with 0, 6, or 100 mM of HU and incubated in a reciprocal shaker (250 rpm) at 30° for 7 hr. The conidiospores were washed with water, conveniently diluted and plated on YUU, and incubated at 30° for 48 hr. Viability was determined as the percentage of colonies on treated plates compared to untreated controls.

Differential display-reverse transcriptase polymerase chain reaction procedure: A total of 1.0×10^7 conidia/ml of *A. nidulans* strain R21 were used to inoculate liquid cultures, which were incubated in a reciprocal shaker at 37° for 16 hr. Mycelia were aseptically transferred to a fresh YG medium in the presence or absence of 25 μM CPT and grown for 1 hr at 37°. *A. nidulans* mycelia were harvested by filtration through a Whatman filter no. 1, washed thoroughly with sterile water, quickly frozen in liquid nitrogen, and disrupted by grinding, and total RNA was extracted with Trizol (Life Technologies). The RNA was reverse transcribed using reverse transcriptase (SuperScript II, GIBCO BRL, Gaithersburg, MD) and the oligonucleotide C2, 5′-GAAGCTGGTAAACAAAAGG-3′, for 30 min at 42°. PCR amplification of the cDNAs was done using the enzyme

TABLE 1

***A. nidulans* strains**

Strains	Genotypes	References
UI224	*pyrG89 yA2; argB2*	Semighini *et al.* (2003)
R21	*pabaA1 yA2*	FGSC A234
GR5	*pyrG89; wA3; pyroA4*	FGSC A773
AAH14	*pyrG89 pabaA1 yA2; argB2;* Δ*uvsB*	Hofmann and Harris (2000)
MV3	*pyrG89 yA2; argB2;* Δ*npkA::pyrG*	This work
MV4	*pyrG89 yA2; argB2;* Δ*npkA::pyrG; alcA::npkA*	This work
SSNI30	*pyrG89;* Δ*argB:trpC*Δ*B; pyroA4;* Δ*pclA::argB veA1*	Schier and Fischer (2002)
A781	*wA2; nimA5*	FGSC
FRY20	*nimX*^{Cdc2AF}*; wA3; pyrg89; pyroA4; pyr4*$^{+}$	Ye *et al.* (1996)
ASH273	*pyrG89 pabaA1 yA2; uvsD153*	De Souza *et al.* (1999)
A776	*pabaA1; acrA1; bimE7; riboB2; chaA1*	FGSC
JL001	*yA2;* Δ*uvsB;* Δ*npkA::pyrG*	This work
JL002	*yA2; bimE7;* Δ*npkA*	This work
JF001	*yA2;* Δ*npkA::pyrG; uvsD153*	This work
APK35	*ankA; pabaA1*	Kraus and Harris (2001)
JF002	*yA2;* Δ*npkA ankA*	This work

FGSC, Fungal Genetics Stock Center.

Taq-Gold (Applied Biosystems, Foster City, CA) and the oligonucleotide C2, using the following PCR conditions for amplification: 95° for 3 min, 42° for 1 min, 72° for 1 min for 35 cycles; and 72° for 5 min. The cDNAs were differentially displayed by running a polyacrylamide gel and staining it with silver nitrate. The bands that displayed differential expression were cut from the gel, eluted, and reamplified. They were subsequently cloned into pUC18 and the inserts were sequenced using M13 reverse and forward primers and BigDye terminator cycle sequencing (Applied Biosystems).

DNA manipulations were according to Sambrook *et al.* (1989). A chromosome-specific cosmid library was screened with the amplified 240-bp *npkA* fragment identified in the differential display-reverse transcriptase polymerase chain reaction (DD-RT-PCR) procedure. This fragment was labeled with [α-^{32}P]dCTP using the kit RTS Rad Prime DNA labeling system (GIBCO-BRL). This screen yielded a single clone (plate 2, W28A05) located on chromosome I. Both genomic and cDNA fragments were fully sequenced using gene-specific primers and BigDye terminator cycle sequencing (Applied Biosystems).

Molecular cloning, deletion, and construction of a conditional mutant of the *npkA* gene: To delete the *npkA* gene, we used the gene replacement method described by Chaveroche *et al.* (2000). Electrocompetent cells of a transformant carrying pKOBEG (Cm^{R}) and cosmid W28A05 (Amp^{R} Km^{R}), a derivative of pWE15, which carries an *A. nidulans* genomic region encompassing the *npkA* gene, were prepared from a culture containing 0.2% arabinose to induce the expression of the *red* genes. Electrocompetent cells were electroporated with ~100 ng of the gel-purified fragment of pCDA21, generated using the following oligonucleotides: *npkAzeo* (5′-TGC GGTGAGTGCATGTCGAAAATACTGAGCTCAGTGCTTAT TGTTGGACGggaattctcagtcctgctcc-3) and *npkApyr* (5′-AAAGA ACATACAAGAAGTAAAATGCAGGAATCATCGCCTGCGA CGAAACCgaattcgcctcaaacaatgc-3′). These oligonucleotides have 50 bp of homology to the 5′ or 3′ noncoding region of the *A. nidulans npkA* (uppercase) followed by 20 bp of homology to the zeocin resistance or *A. fumigatus pyrG* genes carried by pCDA21 (lowercase), respectively. Shocked cells were plated on LB medium containing ampicillin (50 μg/ml) and zeocin (50 μg/ml) and incubated at 30°. Transformants were colony purified once at 42° on medium containing ampicillin and zeocin and then screened for chloramphenicol resistance to test loss of pKOBEG. The resulting cosmids were further characterized by restriction enzyme digestion and PCR analysis. Transformation of *A. nidulans* strains (pyrG89 recipient strains; UI224 and MV3, Table 1) was according to the procedure of Osmani *et al.* (1987) using 5 μg of circular pnpkApyrG or *palcA::npkA* DNA, respectively. Transformants were scored for their ability to grow on minimal medium as the sole carbon source.

For the construction of *alcA(p)::npkA* fusion genes, the following procedure was used. The plasmid pRCP29, which possesses the *argB* selectable marker and the *alcA* promoter, was cut at the unique *Sma*I site, blunted with Klenow fragment, and dephosphorylated with calf intestinal phosphatase (CIP). The *npkA* gene was amplified by PCR using the primers anpk5, 5′-GATGCGCGGCCGCTCGACCTCTAAATCCAGATG-3′ (the underlined region shows a single *Not*I site that was introduced at the 5′-end of the *npkA* gene), and anpk3, 5′-ACGCTGAATT CCCTAAATTTTGAAGGAGAAAA-3′. The fragment containing the *npkA* ORF sequence was gel purified and ligated into plasmid pRCP29. The resulting plasmid (*palcA::npkA*) was digested with *Not*I and dephosphorylated with CIP, and the hemagglutinin 3 (HA3) fragment (Hopp *et al.* 1988) with bordering *Not*I sites was ligated into this plasmid. The *alcA-(p)::HA3::npkA* junction was sequenced to confirm sequence and orientation. This contruct was used to transform the Δ*npkA* (*argB2*) strain to arginine prototrophy.

RNA isolation: A total of 1.0×10^{7} conidia/ml were used to inoculate 50 ml of liquid cultures that were incubated in a reciprocal shaker at 37° for 16 hr. Mycelia were aseptically transferred to fresh YG medium in the presence or absence of drugs for 1, 2, 4, and 8 hr. The following concentrations of chemicals were used: 25 μM of CPT, 0.5 μg/ml of 4-nitroquinoline oxide (4-NQO), 0.003% of MMS, and 0.6 μg/ml of bleomycin (BLEO). Mycelia were harvested by filtration through no. 1 Whatman filter, washed thoroughly with sterile water, quickly frozen in liquid nitrogen, disrupted by grinding, and total RNA was extracted with Trizol (Life Technologies). Ten micrograms of RNA from each treatment was then frac-

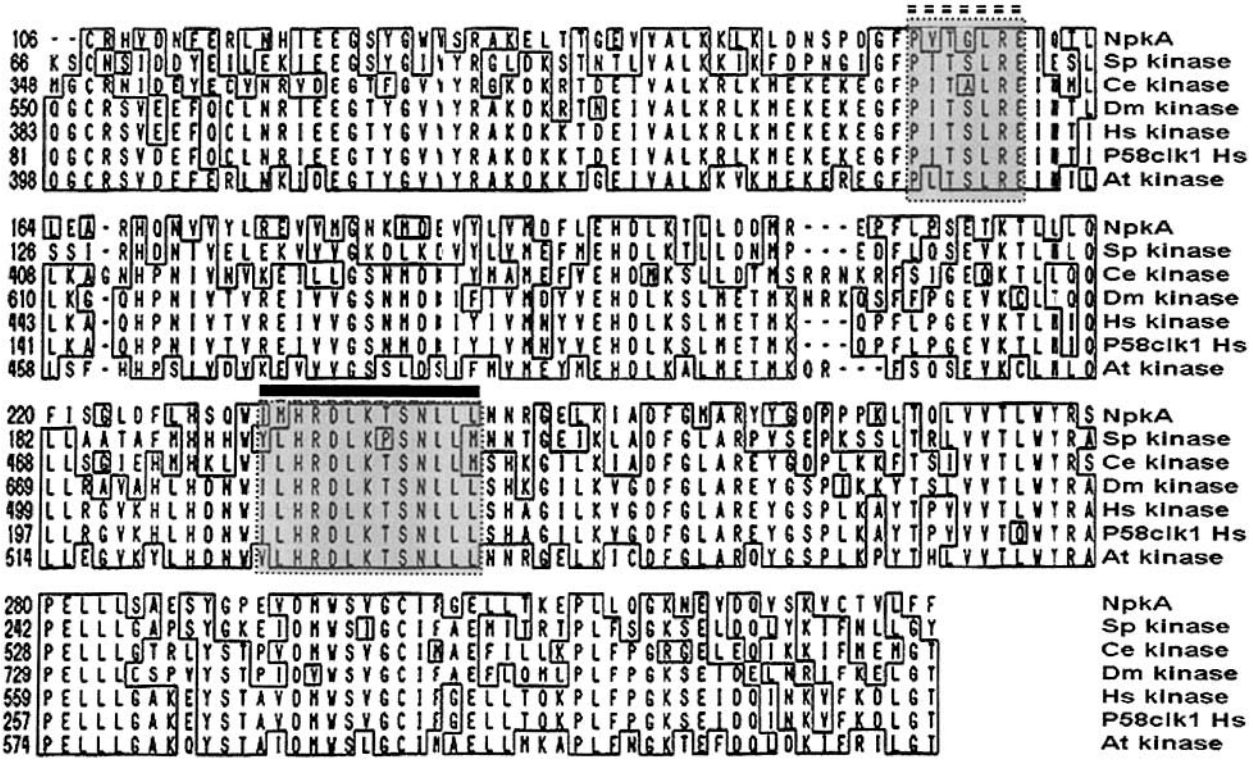

FIGURE 1.—The NpkA protein shows high identity with several PITSLRE protein kinases. Alignment of the serine/threonine kinase domain deduced NpkA sequence (anpk) with *Schizosaccharomyces pombe* cell division cycle 2 homolog (gi/19112531; Sp kinase; 57% identity), *Caenorhabditis elegans* Cdc2 putative kinase (gi/17531375; Ce kinase; 55% identity), *Drosophila melanogaster* PITSLRE gene product (gi/21355495; Dm kinase; 53% identity), *Homo sapiens* Cdc2-like1 PITSLRE protein (gi/16332364; Hs kinase; 58% identity), *H. sapiens* p58 galactosyltransferase-associated protein kinase (gi/107255; p58clk1Hs; 58% identity), and *A. thaliana* putative protein kinase (gi/15220477; At kinase; 56% identity). Thick solid bar, serine/threonine kinase signature; dotted lines, PITSLRE domain.

tionated in 2.2 M formaldehyde and 1.2% agarose gel, stained with ethidium bromide, and then visualized with UV light. The presence of intact 28S and 18S ribosomal RNA bands was used as a criterion to assess the integrity of the RNA. RNAse-free DNAse treatment was done as previously described by SEMIGHINI *et al.* (2002).

Real-time PCR reactions: All the PCR and RT-PCR reactions were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystem). Taq-Man EZ RT-PCR kits (Applied Biosystems) were used for RT-PCR reactions. The thermal cycling conditions comprised an initial step at 50° for 2 min, followed by 30 min at 60° for reverse transcription, 95° for 5 min, and 40 cycles at 94° for 20 sec and 60° for 1 min. The Taq-Man Universal PCR master mix kit was used for PCR reactions. The thermal cycling conditions comprised an initial step at 50° for 2 min, followed by 10 min at 95°, and 40 cycles at 95° for 15 sec and 60° for 1 min. The reactions and calculations were performed according to SEMIGHINI *et al.* (2002). The following primers and Taq-Man fluorescent probes (Applied Biosystems) were used in this work: for β-tubulin (*tubC*), tubCfw, 5′-CGGAAACTGGCCGTCAA TAT-3; tubCrv, 5′-GGGCAAACCCGACCATAAA-3′; tubCprobe, 6FAM-5′-TCCCTTCCCGCGGTTGCATTT-TAMRA; and for *npkA*, npkAfw, AATGGCACGCTACTACGGAGA; npkArv, 5′-GCG GTACCAAAGCGTCACA-3′; npkAprobe, TET-5′-CCTCCGCC AAAACTAACGCAACTCG-3′-TAMRA (6FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2′,7′-tetrachlorofluorescein; and TAMRA, 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine).

RESULTS

Isolation of the *A. nidulans npkA* gene that encodes a p34^{Cdc2}-related serine/threonine kinase: In an attempt to identify genes that are transcriptionally induced upon exposure to CPT, we performed a modified version of the mRNA differential display-reverse transcriptase polymerase chain reaction method of LIANG and PARDEE (1992). Two independent differential display experiments were performed with RNA from noninduced and CPT-induced mycelia, using a single nonanchored (random) primer. We identified several DNA fragments that were either repressed or induced in the presence of CPT (M. A. CASTRO DANI, data not shown). Among them was a DNA fragment of ~240 bp, *cig1* (*c*amptothecin-*i*nduced *g*ene; data not shown). Sequence analysis of this fragment showed that *cig1* corresponded to a gene that encodes a serine/threonine kinase (data not shown).

The *cig1* fragment was used to screen an *A. nidulans* chromosome-specific cosmid library. Sequence analysis of a genomic clone derived from chromosome I confirmed that the DNA fragment corresponded to a gene encoding a putative serine/threonine kinase, which we

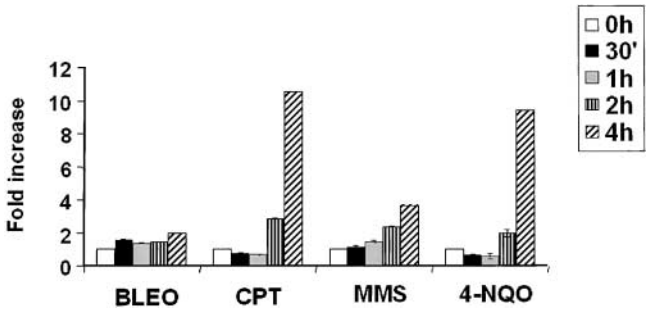

FIGURE 2.—Fold increase in *npkA* RNA levels in response to DNA-damaging agents. The measured quantity of the *npkA* mRNA in each of the treated samples was normalized using the C_T values obtained for the *tubC* RNA amplifications run in the same plate. The relative quantitation of *npkA* and tubulin gene expression was determined by a standard curve (*i.e.*, C_t values plotted against the logarithm of the DNA copy number). Results of four sets of experiments were combined for each determination; means ± standard deviations are shown. The values represent the number of times the genes are expressed compared to the wild-type control grown without any drug (represented absolutely as 1.00).

named *npkA*. When *cig1* was used as a probe in a Northern blot, it recognized a single transcript of ~1.3 kb (Figure 3C). The *npkA* gene does not have any introns, as determined by RT-PCR experiments using appropriate combinations of primers (data not shown). The *npk*A coding region is 1011 nucleotides long, encoding a predicted translation product 336 amino acids long with a calculated molecular weight of ~38,409 D and a calculated isoelectric point of 5.99. Southern blots of total DNA using *npkA* as a probe and searches of the *A. nidulans* genome database (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) indicated that *npkA* is a single-copy gene in the *A. nidulans* genome (data not shown).

The predicted *npkA* protein product showed ~55% identity with the carboxy-terminal domains of several kinases related to p34^{Cdc2} (Cdc2-related kinases; Figure 1). All these Cdc2-related kinases belong to the PITSLRE kinase superfamily (for a review, see LAHTI *et al.* 1995). The PITSLRE domain in NpkA is from amino acids 153–160; there are two substitutions in the PITSLRE domain: an isoleucine by a valine and a serine by a glycine (Figure 1). NpkA shows ~40% identity at the carboxy terminus with the *A. nidulans* NimX^{Cdc2} (data not shown). Additional features of NpkA include a protein kinase domain (amino acids 112–336), including a serine/threonine protein kinase active-site signature (amino acids 232–244). In addition, the predicted sequence includes putative cAMP- and cGMP-dependent protein kinase phosphorylation sites (amino acids 71–74), protein kinase C phosphorylation sites (amino acids 3–5 and 333–335), and casein kinase II phosphorylation sites (amino acids 81–84, 133–136, 162–165, 199–202, and 222–225).

We analyzed the mRNA expression of the *npkA* gene using real-time RT-PCR. Expression was analyzed in the presence of CPT, as well as other DNA-damaging agents, such as MMS, 4-NQO, and BLEO. Wild-type *A. nidulans* was grown in the absence of any drug, transferred to a specific concentration of one of the DNA-damaging agents for 1, 2, 4, and 8 hr, and then RNA was isolated and analyzed for the expression of the *npkA* gene (Figure 2). The *npkA* mRNA expression was induced ~11-, 4-, and 10-fold after 4 hr growth in the presence of CPT, MMS, and 4-NQO, respectively; no increase in *npkA* mRNA levels was observed with the BLEO treatment (Figure 2). Interestingly, this induction is slow when compared to other *A. nidulans* DNA-damage-induced genes whose expression has been analyzed by real-time RT-PCR, such as *scaA*^{NBS1}, *mreA*^{MRE11}, and *rad50* (SEMIGHINI *et al.* 2003). DD-RT-PCR had detected the *cig1* DNA fragment in RNA isolated from cultures grown in the presence of CPT for 1 hr (data not shown). Real-time RT-PCR experiments detected a 3-fold increase in *npkA* RNA levels after 2 hr of growth in the presence of CPT. While the quantitative differences in the two experiments may be due to intrinsic peculiarities of each assay used, both experiments indicate that *npKA* is induced at the transcriptional level in the presence of CPT and other DNA-damaging agents, suggesting that *npkA* could be involved in the *A. nidulans* DNA damage response.

Construction of a conditional *npkA* mutant strain: To investigate the function of the *npkA* gene, an *A. nidulans* strain carrying a deletion of the *npkA* gene was generated using the method described by CHAVEROCHE *et al.* (2000) for *A. nidulans*. This method relies on the ability of *E. coli* strains expressing the λ *red* genes to carry out homologous recombination between homologous sequences of <60 bp. Cosmid W28A05, which contains the *npkA* gene and flanking genomic region, was introduced into an *E. coli* strain carrying pKOBEG. The resulting strain was transformed with a PCR product obtained through amplification of the *zeo/pyrG* cassette of pCDA21 using oligonucleotides with 50 bp of homology to the 5′- or 3′-end of the *npkA* coding region and 20 bp of homology in the *zeo/pyrG* cassette. Several hundred Zeo^R transformants were obtained and characterization of some of them by restriction enzyme and PCR analysis showed that most resulted from the expected *npkA* allelic exchange on cosmid W28A05. Protoplasts of *A. nidulans* strain UI224 were transformed using one of these plasmids (NPK1) in its circular form. Several transformants were obtained by their ability to grow in minimal medium without uracil and uridine but supplemented with arginine. Allelic replacement was obtained in at least one of these transformants (MV3 strain) as confirmed by Southern blot analysis (Figure 3A). The *A. nidulans npkA* deletion strain showed no growth defects or changes in developmental patterns (data not shown). In addition, the MV3 strain was neither more

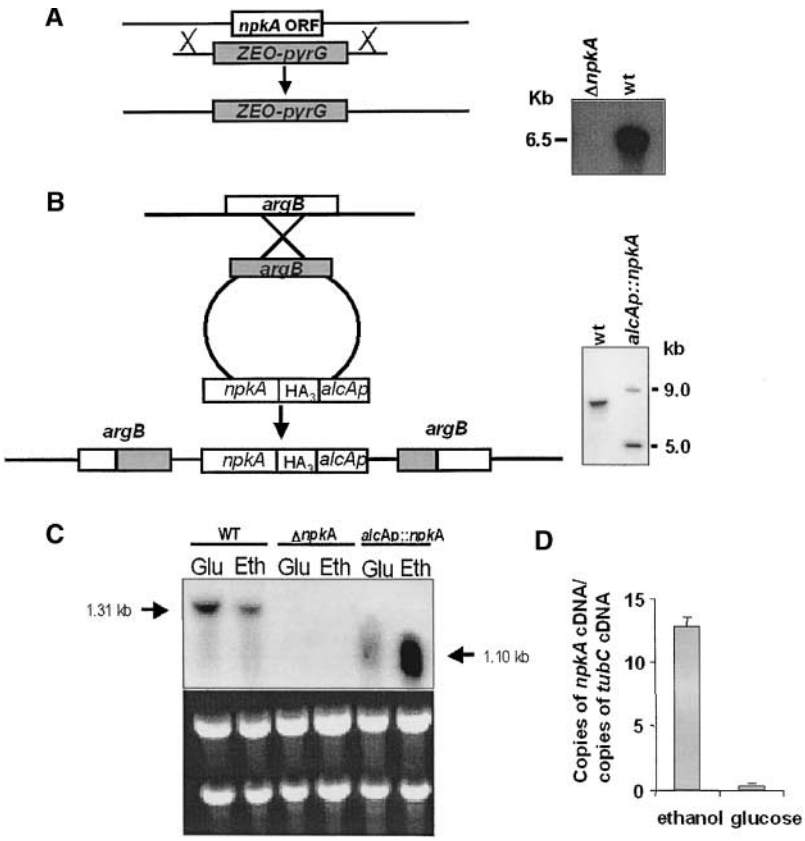

FIGURE 3.—Construction of deletion and overexpression strains. (A, left) The *npkA* gene was deleted by using the *zeo-pyrG* cassette (see text for details). (A, right) Southern analysis of the MV3 (Δ*npkA*) and wild-type strains. DNA from MV3 and wild-type strains were isolated and cleaved with enzyme *Eco*RI; the *npkA* gene was used as hybridization probe. (B, left) Diagram showing the integration of the plasmid p*alcA::HA3::npkA* into the *argB* locus. (B, right) Southern analysis of the integration of the plasmid p*alcA::HA3::npkA* into the *argB* locus. DNA from the wild-type and MV4 strains was isolated and cleaved with enzyme *Bam*HI; the *argB* gene was used as hybridization probe. (C) Northern blot analysis of the wild-type, MV3, and MV4 strains grown in the presence of either MC + 20 g/liter of glucose or MC + 200 mM of ethanol; *npkA* gene was used as a probe. The arrows indicate the respective *nkpA* transcript size in the wild-type and MV4 strains. (D) Real-time RT-PCR of the *npkA* mRNA expression of the wild-type strain grown in the presence of either MC + 200 mM ethanol or 20 g/liter glucose. The measured quantity of the *npkA* mRNA in each of the treated samples was normalized using the C_T values obtained for the *tubC* RNA amplifications run in the same plate. The relative quantitation of *npkA* and tubulin gene expression was determined by a standard curve (*i.e.*, C_t values plotted against the logarithm of the DNA copy number). Results of four sets of experiments were combined for each determination; means ± standard deviations are shown. The values represent the copies of the *npkA* cDNA divided by the copies of the *tubC* cDNA.

sensitive nor more resistant to DNA-damaging agents such as BLEO, CPT, MMS, and 4-NQO. Furthermore, it displayed nuclear kinetics, septation, and growth rate comparable to the wild-type strain (data not shown). These results suggest that either NpkA is not involved in these processes or other kinases are able to compensate for the lack of NpkA.

We used the MV3 strain to construct a conditional mutant by fusing the *npkA* ORF to the *A. nidulans alcA* alcohol dehydrogenase promoter in pRCP29, a derivative of the vector pAL5 (WARING *et al.* 1989) that has *argB* as a selectable marker. A 3xHA epitope was fused in frame at the N terminus of the *npkA* ATG (for details, see MATERIALS AND METHODS). This construction (p*alcA::HA3::npkA*) was transformed into the MV3 strain and transformants were selected for arginine prototrophy and by Southern blot analysis. The p*alcA::HA3::npkA* plasmid had integrated at the endogenous *A. nidulans argB* locus in one of these transformants (strain MV4; Figure 3B). Northern blot analysis and real-time RT-PCR confirmed that the *npkA* gene in MV4 was regulated by the *alcA* promoter, since expression was repressed on glucose, and induced at least 26-fold on ethanol (Figure 3, C and D). The differences in the size of mRNA transcripts recognized by the *npkA* probe in the wild-type (~1.3-kb) and p*alcA::HA3::npkA* (~1.1-kb) strains in the Northern blot are consistent with the absence of the leader and tail sequences from the latter (Figure 3C).

Overexpression of the *npkA* gene in strain MV4, like deletion of *npkA*, did not cause any measurable growth defects, changes in developmental patterns, or sensitivity to BLEO, CPT, MMS, and 4-NQO. In addition, nuclear kinetics, septation, and growth rate remained unchanged in this strain (data not shown).

The *npkA* deletion can suppress Δ*uvsB*^{ATR} and *uvsD153*^{ATRIP} sensitivity to HU: In several eukaryotic organisms, DNA damage checkpoint activation is controlled by the conserved family of ATM/ATR kinases (SHILOH 2001; NYBERG *et al.* 2002), which includes *A. nidulans* UvsB^{ATR} (DE SOUZA *et al.* 1999). Recently, CLIBY *et al.* (2002) have shown that ATR is important in responding to the replication-associated DNA damage from topoisomerase poisons. Since *npkA* RNA levels are increased by treatment with the antitopoisomerase I poison CPT, we investigated whether *npkA* gene function was related to *uvsB*^{ATR} activity. First, we checked if the induction of *npkA* transcription in response to DNA damage is dependent on the *uvsB*^{ATR} gene. The *uvsB*^{ATR} deletion mutant and wild-type strains were grown in the absence of any drug, transferred to 25 μM of CPT for 4 hr, and RNA was isolated and analyzed for the expression of the *npkA* gene by real-time RT-PCR. Under these

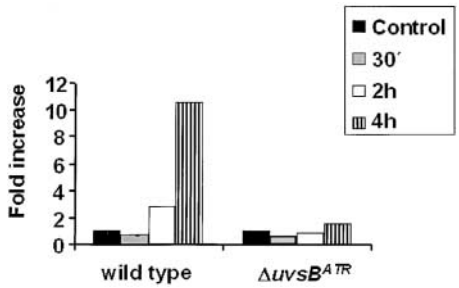

FIGURE 4.—Expression of *npkA* mRNA is dependent on the *uvsB*^{ATR} gene when *A. nidulans* is grown in the presence of CPT. The wild-type and Δ*uvsB* strains were grown in YG medium for 12 hr at 37° and then transferred to YG or YG + 25 μM of CPT. RNA was extracted, DNAse treated, and RT-PCR reactions were run. The measured quantity of the *npkA* mRNA in each of the treated samples was normalized using the C_T values obtained for the *tubC* RNA amplifications run in the same plate. The relative quantitation of *npkA* and tubulin gene expression was determined by a standard curve (*i.e.*, C_t values plotted against logarithm of the DNA copy number). Results of four sets of experiments were combined for each determination; means ± standard deviations are shown. The values represent the number of times the genes are expressed compared to the wild type or Δ*uvsB* grown without any drug (represented absolutely as 1.00).

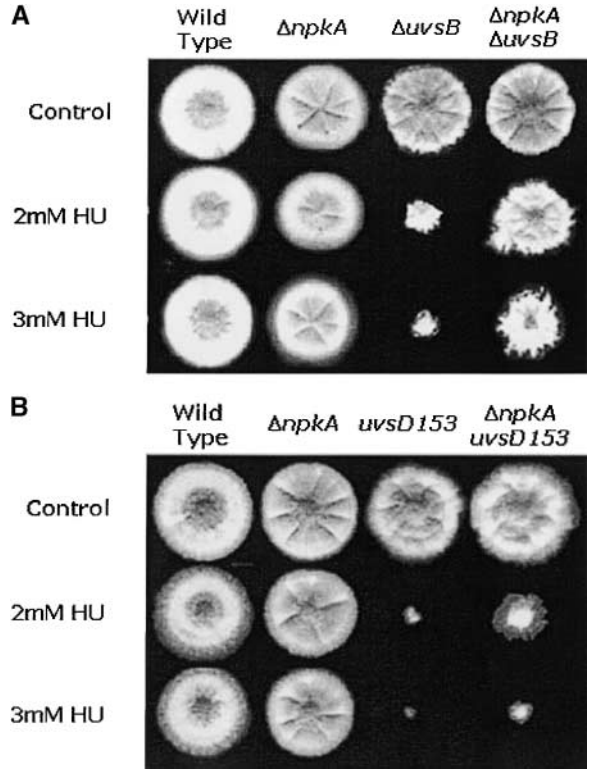

FIGURE 5.—Growth phenotypes of the Δ*npkA* Δ*uvsB*^{ATR} and Δ*npkA uvsD153*^{ATRIP} double mutants. (A) Strains GR5 (wild type), MV3 (Δ*npkA*), AAH14 (Δ*uvsB*^{ATR}), and JL001 (Δ*npkA* Δ*uvsB*^{ATR}) were grown for 72 hr at 37° in YUU medium in the presence or absence of HU. (B) Strains GR5 (wild type), MV3 (Δ*npkA*), ASH273 (*uvsD153*^{ATRIP}), and JF001 (Δ*npkA uvsD153*^{ATRIP}) were grown for 72 hr at 37° in YUU medium in the presence or absence of HU.

conditions, *npkA* mRNA levels were induced ~10-fold in the control strain, whereas no induction of *npkA* transcription was detectable in the *uvsB* mutant (Figure 4). These results show that the highly induced transcript levels of the *npkA* gene in the presence of CPT require *uvsB*^{ATR}.

To analyze the genetic interactions of *npkA* and *uvsB*^{ATR}, we constructed an Δ*npkA* Δ*uvsB*^{ATR} double mutant (strain JL001). This JL001 strain was inhibited to the same degree as the *uvsB*^{ATR} strain when grown in the presence of CPT, 4-NQO, and MMS (data not shown). However, in the double mutant, the Δ*npkA* mutation partially suppressed the HU sensitivity seen in the Δ*uvsB*^{ATR} mutant (Figure 5A).

Human ATR exists in a stable complex with a protein called ATRIP (CORTEZ *et al.* 2001). Fission yeast Rad3 and budding yeast Mec1 ATR orthologs also form similar complexes with the ATRIP-related factors Rad26 and Ddc2/Lcd2/Pie1, respectively (PACIOTTI *et al.* 2000; WOLKOW and ENOCH 2002). In human cells, ATR localizes with ATRIP in nuclear foci after damage, suggesting that the ATR-ATRIP complex may be recruited to the sites of DNA damage (CORTEZ *et al.* 2001). In *A. nidulans*, the Atrip/Rad26 homolog is UvsD (DE SOUZA *et al.* 1999). To find out about possible genetic interactions between *npkA* and *uvsD153*^{ATRIP}, we constructed an Δ*npkA uvsD153*^{ATRIP} double mutant (strain JF001). As with *uvsB*^{ATR}, the growth of this Δ*npkA uvsD153*^{ATRIP} strain in the presence of CPT, 4-NQO, and MMS was similar to the growth of the *uvsD153*^{ATRIP} single-mutant strain (data not shown). However, as with *uvsB*^{ATR}, *npkA* partially suppressed the HU sensitivity of the *uvsD153*^{ATRIP} mutation (Figure 5B).

Taken together these results suggest that NpkA genetically interacts with the UvsB^{ATR} and UvsD^{ATRIP} complex during DNA damage caused specifically by HU.

HU is an inhibitor of ribonucleoside diphosphate reductase, the rate-limiting step enzyme in deoxyribonucleotide triphosphate (dNTP) biosynthesis. Depletion of dNTPs activates the DNA replication checkpoint, which slows progression through S-phase (DESANY *et al.* 1998). Furthermore, initiation of DNA replication in the presence of high levels of HU causes DNA DSBs (MERRILL and HOLM 1999). HU is an effective inhibitor of DNA synthesis in *A. nidulans* (BERGEN and MORRIS 1983). To better understand the Δ*npkA* suppression of HU sensitivity in the ATR and ATRIP mutants, we examined the ability of the strains Δ*npkA* Δ*uvsB*^{ATR} and Δ*uvsB*^{ATR} to survive a transient period of growth in the presence of HU. According to ALLEN *et al.* (1994), this acute treatment causes severe lethality in mutants that are specifically defective in the DNA replication checkpoint but not in mutants affected in DNA repair or in other DNA damage checkpoints. Thus, on the basis of HU hypersensitivity it is possible to distinguish between a mutant involved in the S-phase checkpoint and a mutant involved in DNA repair. Accordingly, two different assays

TABLE 2

***A. nidulans* double mutant Δ*uvsB* Δ*npkA* has intact intra-S-phase checkpoint**

	HU (mM)		
Strain	0	6	100
Wild type	63.0 ± 12.0	8.5 ± 0.7	1.5 ± 0.7
AAH14 (Δ*uvsB*)	61.5 ± 7.8	16.5 ± 2.0[a]	15.5 ± 0.7[e]
MV3 (Δ*npkA*)	68.5 ± 2.1	26.0 ± 2.8[b]	2.0 ± 2.8
JL001 (Δ*uvsB* Δ*npkA*)	68.0 ± 5.6	4.0 ± 2.6	0 ± 0
A776 (*bimE*)	39.3 ± 2.5	23.3 ± 5.9	0 ± 0
JL002 (*bimE* Δ*npkA*)	92.0 ± 1.0	72.0 ± 9.8[c]	8.7 ± 2.0[f]
APK35 (*ankA*)	94.0 ± 4.7	25.3 ± 6.7	0.3 ± 0
JF002 (*ankA* Δ*npkA*)	77.0 ± 3.5	64.2 ± 3.7[d]	11.5 ± 4.6[g]

Percentage of germlings that had two or more nuclei after the HU incubation were scored as germlings that disrupted the S-phase blockage. All the results are the average of determinations from four independent experiments with 100 germlings being evaluated in each. The data are the average of three repetitions and means are ± standard deviation. Germlings that disrupted intra-S-phase checkpoint were scored. The results were expressed by the average ± standard deviation. Statistical differences were determined by ANOVA followed, when significant, by Newman-Keuls Multiple Comparison Test, using Sigma Stat statistical software (Jandel Scientific). $P < 0.05$ was considered statistically significant.

[a] Significantly different from JL001 and wild-type strains ($P < 0.001$).
[b] Significantly different from JL001, JL002, and JF002 strains ($P < 0.001$).
[c] Significantly different from A776 strain ($P < 0.001$).
[d] Significantly different from APK35 strain ($P < 0.001$).
[e] Significantly different from MV3, JL001, and wild-type strains ($P < 0.001$).
[f] Significantly different from MV3 and A776 strain ($P < 0.001$).
[g] Significantly different from MV3 strain ($P < 0.001$).

were used to distinguish these differences. In the first one, the intra-S-phase checkpoint assay, the strains were incubated in 6 or 100 mM of HU for 5–7 hr. The number of nuclei was assessed by DAPI staining and if the germlings had two or more nuclei after the HU incubation, they were scored as defective in S-phase arrest (Table 2). In the second one, the DNA replication checkpoint assay, the germling viability was assessed after incubation for 7 hr in the presence and absence of 6 or 100 mM of HU (Table 3).

Interestingly, the Δ*npkA* strain showed to have defects in the S-phase arrest at 6 mM while the Δ*uvsB*^{ATR} germlings displayed defects at 6 and 100 mM (Table 2). However, the double-mutant Δ*uvsB* Δ*npkA* (JL001) strain has shown an intact intra-S-phase checkpoint at both 6 and 100 mM. The Δ*npkA* (MV3) strain has shown an impaired response in the DNA replication checkpoint at 6 mM but not at 100 mM. The DNA replication checkpoint is impaired in the Δ*uvsB*^{ATR} strain at both 6 and 100 mM of HU (Table 3). Remarkably, the double-mutant Δ*uvsB* Δ*npkA* (JL001) strain has shown a synergism with decreased viability at 6 mM but a comparable viability with the Δ*uvsB*^{ATR} (AAH14) strain at 100 mM HU. These results suggest that there is a defect in both the intra-S-phase and DNA replication checkpoints due to the *npkA* inactivation when DNA replication is slowed at 6 mM HU. Furthermore, the *uvsB*^{ATR} is involved in both intra-S-phase and DNA replication checkpoints in *A. nidulans*. In addition, once more a genetic interaction is observed between *npkA* and *uvsB*^{ATR} since the Δ*npkA* can suppress the *uvsB*^{ATR} intra-S-phase checkpoint deficiency and the double mutant Δ*npkA uvsB*^{ATR} showed decreased viability after incubation at 6 mM HU.

Since the ATR pathway can also respond to UV light, an agent that interferes with the function of replication forks (Nyberg *et al.* 2002; Osborn *et al.* 2002), we checked UV sensitivities of nondividing (quiescent) and dividing (germinating) Δ*npkA* Δ*uvsB*^{ATR} cells to UV irradiation. When UV irradiation was applied to quiescent conidia, the Δ*npkA* Δ*uvsB*^{ATR} double-mutant strain was not more sensitive than either of the single-mutant strains, Δ*npkA* and Δ*uvsB*^{ATR}, to UV irradiation (Figure 6). Likewise, germinating Δ*npkA* Δ*uvsB*^{ATR} conidia were as sensitive to UV irradiation as the Δ*uvsB*^{ATR} (Figure 6). In the latter experiment, conidiospores were first allowed to germinate for 4.5 hr before UV irradiation was applied, by which time they had entered the first cell cycle. These results show that Δ*uvsB*^{ATR} germinating conidiospores are more sensitive than quiescent conidiospores to UV irradiation and indicate that the Δ*npkA* mutation does not affect the *uvsB*^{ATR} UV light sensitivity when conidiospores are either quiescent or germinating.

Possible genetic interactions with *ankA*^{wee1} and *bimE*^{APC1}: Ye *et al.* (1996) have shown the existence of two S-phase checkpoint regulatory systems that control initiation in mitosis. One, which involves both Y15 phosphorylation of p34^{Cdc2} and BimE, prevents initiation of mitosis when DNA replication is arrested and the other restrains mitosis via Y15 phosphorylation of p34^{Cdc2} when DNA replication is slowed. Thus, we investigated possible genetic

TABLE 3

***A. nidulans uvsB*^{ATR} is not involved in the DNA replication checkpoint**

Strain	HU (mM)	
	6	100
Wild type	100.0 ± 0	100.0 ± 0
AAH14 (Δ*uvsB*)	74.6 ± 12.8[a]	74.7 ± 10.3[d]
MV3 (Δ*npkA*)	86.4 ± 6.8[b]	98.0 ± 3.3[e]
JL001 (Δ*uvsB* Δ*npkA*)	44.3 ± 7.3	59.1 ± 5.1
A776 (*bimE*)	94.8 ± 6.5	97.8 ± 4.4
JL002 (*bimE* Δ*npkA*)	98.3 ± 3.5	54.7 ± 16.1
APK35 (*ankA*)	50.4 ± 9.8	100.0 ± 0
JF002 (*ankA* Δ*npkA*)	95.8 ± 6.4[c]	98.0 ± 4.9

Viability was determined as the percentage of colonies on treated plates compared to untreated controls. All the results are the average of determinations from four independent experiments. The data are the average of three repetitions and means are ± standard deviation. Statistical differences were determined by ANOVA followed, when significant, by Newman-Keuls Multiple Comparison Test, using Sigma Stat statistical software (Jandel Scientific). $P < 0.05$ was considered statistically significant.

[a] Significantly different from JL001 and wild-type strains ($P < 0.001$).

[b] Significantly different from APK35 and JL001 strains ($P < 0.001$).

[c] Significantly different from APK35 strain.

[d] Significantly different from MV3 and wild-type strains ($P < 0.001$).

[e] Significantly different from JL001 and JL002 strains ($P < 0.001$).

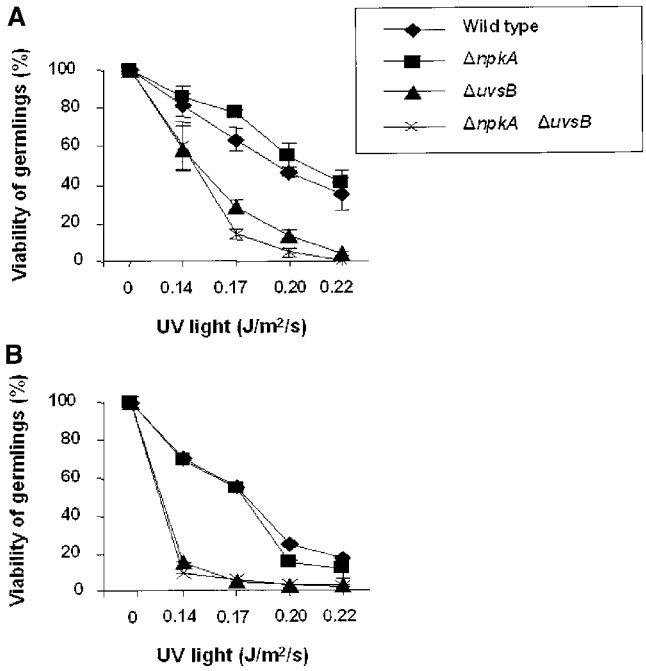

FIGURE 6.—The Δ*npkA* mutation cannot suppress the UV-light sensitivity of the Δ*uvsB* mutant in quiescent (A) and germinating (B) conidiospores. Viability of germlings was scored after exposure to UV light. Viability was determined as the percentage of colonies on treated plates compared to untreated controls. The results were expressed by the average of three independent experiments and means are ± standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA) followed, when significant, by Newman-Keuls Multiple Comparison Test, using Sigma Stat statistical software (Jandel Scientific). The Δ*uvsB* and Δ*uvsB* Δ*npkA* strains were significantly different from wild type and Δ*npkA*; $P < 0.01$.

interactions among *npkA* and *ankA*^{wee1} and *bimE*^{APC1}. Accordingly, we constructed double mutants Δ*npkA bimE* (strain JL002) and Δ*npkA ankA* (strain JF002). The double-mutant strain Δ*npkA bimE* was inhibited to the same extent as the corresponding parental strains when grown in the presence of CPT, 4-NQO, MMS, and HU (data not shown). However, the double mutant Δ*npkA ankA* is more sensitive than the parental strains to 4-NQO, MMS, and CPT (Figure 7) and as resistant to HU as MV3 and APK35 (data not shown).

We also verified if the S-phase checkpoints were intact in these double-mutant strains (Tables 2 and 3). The *bimE* mutant has an impaired intra-S-phase checkpoint at 6 mM but an intact one at 100 mM while the DNA replication checkpoint is intact at both 6 and 100 mM (Tables 2 and 3). There is a synergism in the double-mutant Δ*npkA bimE*^{APC1} (JL002) strain during the intra-S-phase checkpoint with more germlings than the parental strains having two nuclei at both 6 and 100 mM HU (Table 2). Curiously, there is an intact DNA replication checkpoint in the JL002 strain at 6 mM but a synergism with a decrease in viability at 100 mM (Table 3). The *ankA* mutant strain (APK35) has impaired and intact intra-S-phase checkpoints at 6 and 100 mM, respectively. The double-mutant Δ*npkA ankA* strain (JF002) has shown an increase in the number of germlings with two nuclei at both 6 and 100 mM (Table 2). However, the JF002 strain has not shown any defects in the DNA replication checkpoint (Table 3). These results strongly suggest that there are genetic interactions between *npkA* and *bimE* and *npkA* and *ankA* during S-phase checkpoints.

DISCUSSION

We have used DD-RT-PCR to isolate cDNAs that correspond to genes induced or repressed by the antitopoisomerase I drug CPT. This drug is an antitopoisomerase I poison and cytotoxic lesions are thought to occur when the advancing replication machinery encounters a CPT-stabilized topoisomerase I-DNA complex (HSIANG *et al.* 1985; LIU 1989, 1994; FROELICH-AMMON and OSHEROFF 1995; GUPTA *et al.* 1995; WANG 2002). The repair of a topoisomerase lesion presents special problems because the strand break is entangled with a covalently bound polypeptide. To restore the integrity of the chromosomal DNA, this polypeptide must be removed. Mutants

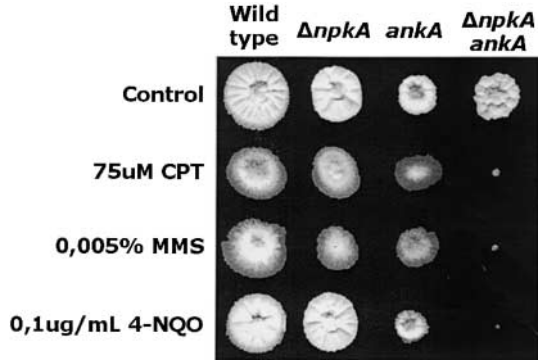

Figure 7.—Growth phenotypes of the Δ*npkA ankA* double mutant. Strains R21 (wild type), MV3 (Δ*npkA*^{wee1}), APK35 (*ankA*^{wee1}), and JF002 (Δ*npkA* Δ*uvsB*^{ATR}) were grown for 72 hr at 37° in YAG medium in the absence (first row) or presence of CPT (second row), MMS (third row), and 4-NQO (fourth row).

defective in DNA repair show increased sensitivity to topoisomerase poisons and recently Liu *et al.* (2002) and Vance and Wilson (2002) reported that in *Saccharomyces cerevisiae* multiple pathways could repair topoisomerase damage. Liu *et al.* (2002) showed that the *MRE11* gene is more critical than the *RAD52* gene for double-strand break repair of CPT lesions.

Using DD-RT-PCR, we isolated a cDNA fragment that corresponds to a gene encoding a protein (NpkA) whose transcription is induced in cells treated with CPT. NpkA exhibits 55% identity in the kinase domain to several members of the Cdc2-related PITSLRE family of protein kinases and thus is a member of the Crk family of kinases (Meyerson *et al.* 1992). Deletion of the *npkA* did not cause any obvious phenotypic changes, suggesting that NpkA is functionally redundant with other kinases. In *A. nidulans, NimX*^{Cdc2} is the only mitotic CDK, as in most eukaryotic cells. NimX^{Cdc2} activation and entry into mitosis requires association of the *nimE* B-cyclin during interphase (Osmani *et al.* 1994). Schier *et al.* (2001) have isolated the pcl-like cyclin PclA, which is involved in asexual sporulation in *A. nidulans*, and recently Schier and Fischer (2002) showed that PclA interacts with NimX^{Cdc2}. A second *A. nidulans* CDK, PhoA, is homologous to the yeast Pho85 kinase and controls developmental responses to phosphorus-limited growth conditions (Bussink and Osmani 1998). Dou *et al.* (2003) identified another nonessential cyclin-dependent kinase, PhoB, which is 77% identical to PhoA. Double mutant Δ*phoA* Δ*phoB* was able to germinate but had a limited capacity for nuclear division, suggesting a cell cycle defect. We have no evidence indicating that an *A. nidulans* cyclin interacts with NpkA. In fact, the double mutant Δ*npkA::pyrG*; Δ*pclA::argB* was viable and no more sensitive than the parental strains to DNA-damaging agents, suggesting that NpkA does not interact with PclA (data not shown).

A. nidulans npkA transcription is induced not only by CPT, but also by treatment with other DNA-damaging agents, such as the alkylating agent MMS and the UV-mimetic agent 4-NQO. All these agents interfere with the function of replication forks and the damage they cause can activate the ATR pathway (Osborn *et al.* 2002). ATM/ATR are members of the family of phosphoinositide 3-kinase-related kinases and key regulators of the DNA damage response (Caspari and Carr 1999; Shiloh 2001). They trigger responses that promote the maintenance of genome integrity by phosphorylating multiple target proteins. *ATR* inhibition results in hypersensitivity to the replication inhibitors HU and aphidicolin (Cliby *et al.* 1998). These observations suggest that *ATR* responds to DNA damage occurring specifically during S-phase. De Souza *et al.* (1999) and Hofmann and Harris (2000) established that *A. nidulans* UvsB^{ATR} is a member of the family of ATM/ATR kinases. These authors suggested that UvsB functions as the central regulator of the *A. nidulans* DNA damage response. On the basis of these observations, we investigated the genetic interaction of *npkA* and *uvsB*^{ATR}. First, we found that the induction of *npkA* expression in response to CPT treatment requires *uvsB*^{ATR}. Second, we constructed a double mutant Δ*npkA* Δ*uvsB*^{ATR} and analyzed its growth in the presence of several DNA-damaging agents. The double mutant was more resistant than the Δ*uvsB*^{ATR} strain to HU. In addition, the Δ*npkA* mutation partially suppressed the HU sensitivity of the *uvsD*^{ATRIP} mutant.

HU stalls replication forks by depleting the dNTP pool. Another type of replication block might be associated with the DNA breaks generated during DNA replication. In theory, DSBs could arise if replication forks pass through nicked DNA or certain repair or recombination intermediates (Osborn *et al.* 2002). Replication-associated DSBs also could be induced by agents such as topoisomerase I poisons (Cliby *et al.* 2002). Cliby *et al.* (2002) demonstrated that ATR kinase function is necessary for both G_2- and S-phase arrests induced by topoisomerase I poisons. These cellular responses to topoisomerase poisons appear to be independent of ATM function. The S-phase checkpoints respond to replicational interference by slowing down DNA replication to allow the damage to be repaired before polymerases encounter more DNA damage (Osborn *et al.* 2002). Deletion of the *ATR* orthologs *MEC1* and *RAD3* in budding and fission yeasts, respectively, eliminates the DNA replication checkpoint (Nyberg *et al.* 2002). Using a Cre/lox-conditional system to study the effect of *ATR* loss, Brown and Baltimore (2003) showed that mammalian *ATR* is an important regulator of checkpoint signaling pathways that phosphorylates Cdc2 in response to ionizing rays and stalled replication. We found that *A. nidulans* UvsB^{ATR} is involved in both intra-S-phase and DNA replication checkpoints. We determined that the Δ*npkA*

mutation could suppress the *uvsB*^{ATR} HU sensitivity by rescuing the intra-S-phase checkpoint in the Δ*uvsB*^{ATR} mutation. Suppression of a null allele is expected to be due to downstream mutations that activate the pathway independent of the original (suppressed) gene product (Prelich 1999). Thus, it is possible that NpkA is positioned downstream from UvsB^{ATR} in the intra-S-phase checkpoint pathway. Furthermore, the Δ*uvsB*^{ATR} Δ*npkA* double mutant showed decreased viability at lower HU concentration in the DNA replication checkpoint, suggesting that UvsB^{ATR} and NpkA function in parallel pathways to allow S-phase progression and/or recovery.

At least two S-phase checkpoint mechanisms control mitosis in *A. nidulans* (Ye *et al.* 1996). The first S-phase checkpoint is activated during DNA replication that has been slowed by addition of HU to a level that does not arrest replication. It responds to the rate of DNA replication and inhibits mitosis via tyrosine phosphorylation of NimX^{Cdc2}. If DNA replication is arrested, a second checkpoint involves BimE^{APC1} (the homolog of the anaphase promoting complex subunit, APC1). This second S-phase checkpoint occurs when DNA replication is completely inhibited by levels of HU higher than that stimulating the prolonged DNA replication checkpoint. This information was obtained through studies of double mutants of *A. nidulans* containing *nimX*^{Cdc2AF} [a mutated version in which the Thr14 is converted to an Ala (A) and Tyr15 to a Phe (F) residue] and the temperature-sensitive mutation *bimE7* (Osmani and Ye 1997). Either the *Cdc2*AF or the *bimE7* mutation alone has a limited capacity to promote mitosis when S-phase is arrested, but, in combination, these two defects allow cells to enter a lethal premature mitosis before completion of DNA replication.

We investigated the possible genetic interactions among NpkA and BimE^{APC1} and NimX^{Cdc2} by constructing the double mutants *bimE*^{APC1} Δ*npkA* and *ankA*^{wee1} Δ*npkA*. The AnkA is the ortholog of the fission yeast tyrosine kinase Wee1p that inhibits phosphorylation of Tyr-15 of NimX^{Cdc2} (Kraus and Harris 2001). As in fission yeast, the DNA damage checkpoint is regulated by tyrosine phosphorylation of NimX^{Cdc2} (Ye *et al.* 1997). The double mutant Δ*npkA ankA* showed to be more sensitive to 4-NQO, MMS, and CPT. These genotoxins interfere with the function of replication forks and the DNA damage response to these agents is mediated via the ATR pathway (Nyberg *et al.* 2002; Osborn *et al.* 2002). It is likely that *nimX*^{Cdc2} and *npkA* function in parallel pathways downstream from *uvsB*^{ATR}, allowing cell cycle arrest upon DNA damage.

In lower and higher levels of HU, both BimE^{APC1} and AnkA showed synergistic interaction with NpkA, suggesting that these genes also function in parallel pathways to allow intra-S-phase checkpoint. Nevertheless, a more complex behavior was seen during DNA replication checkpoint. In lower HU concentrations, BimE^{APC1} has not interacted with NpkA while there is a decrease in the germling viability in the double mutant *bimE*^{APC1} Δ*npkA* at higher HU concentrations, also suggesting that these two genes also function in parallel pathways to allow DNA replication checkpoint. In lower concentrations of HU, Δ*npkA* suppressed the low viability of the *ankA* mutation; however, in high HU concentrations, there is an intact DNA replication checkpoint.

In conclusion, our data are consistent with the possibility that in *A. nidulans* ATR is one of the sensors for replication-mediated DNA damage and we propose that NpkA, together with NimX^{Cdc2} and BimE^{APC1}, monitors S-phase progression and/or recovery in response to DNA damage. Functional redundancy is most likely the reason why deletion of *npkA* did not result in a clear phenotype. However, NpkA appears to be a component of the ATM/ATR signaling pathway because: (i) *npkA* gene expression is dependent on *uvsB*^{ATR} in the presence of camptothecin, (ii) the *npkA* deletion can partially suppress the HU sensitivity of the *uvsB*^{ATR} and *uvsD*^{ATRIP} mutants, and (iii) the double mutant Δ*npkA ankA* is more sensitive to genotoxins that interfere with the function of replication forks.

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