Schizosaccharomyces pombe Cells Lacking the Amino-Terminal Catalytic Domains of DNA Polymerase Epsilon Are Viable but Require the DNA Damage Checkpoint Control

WENYI FENG AND GENNARO D'URSO*

Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101-6129

In *Schizosaccharomyces pombe*, the catalytic subunit of DNA polymerase epsilon (Pol ε) is encoded by $cdc20^+$ and is essential for chromosomal DNA replication. Here we demonstrate that the N-terminal half of Pol ε that includes the highly conserved polymerase and exonuclease domains is dispensable for cell viability, similar to observations made with regard to *Saccharomyces cerevisiae*. However, unlike budding yeast, we find that fission yeast cells lacking the N terminus of Pol ε ($cdc20^{\Delta N-term}$) are hypersensitive to DNA-damaging agents and have a cell cycle delay. Moreover, the viability of $cdc20^{\Delta N-term}$ cells is dependent on expression of $rad3^+$, $hus1^+$, and $chk1^+$, three genes essential for the DNA damage checkpoint control. These data suggest that in the absence of the N terminus of Pol ε, cells accumulate DNA damage that must be repaired prior to mitosis. Our observation that S phase occurs more slowly for $cdc20^{\Delta N-term}$ cells suggests that DNA damage might result from defects in DNA synthesis. We hypothesize that the C-terminal half of Pol ε is required for assembly of the replicative complex at the onset of S phase. This unique and essential function of the C terminus is preserved in the absence of the N-terminal catalytic domains, suggesting that the C terminus can interact with and recruit other DNA polymerases to the site of initiation.

Genetic analysis of yeast has demonstrated that Pol ε is required for chromosomal DNA replication (4, 7, 9, 14, 32, 40). However, its precise function at the replication fork has remained elusive. Based on our earlier observations that *cdc20* mutants ($cdc20^+$ encodes the catalytic subunit of Pol ε in fission yeast) show a cell cycle arrest with a 1C DNA content, we proposed that Pol ε is necessary during the initiation of DNA replication (14). Consistent with this hypothesis, a chromatin immunoprecipitation assay has demonstrated that Pol ε associates with replication origins in budding yeast (3, 27). Moreover, the observation that Pol ε remains associated with replication forks following initiation suggests that it also participates directly in chain elongation (3).

In addition to its role in DNA replication, Pol ε has also been implicated in DNA repair. Pol ε from human cells has been shown to function in nucleotide excision repair in vitro (39) and has been identified as a component of a high-molecular-weight complex that catalyzes recombinational repair of DNA double-strand breaks in vitro (18). Genetic analysis of *Saccharomyces cerevisiae* also supports a role for Pol ε in DNA double-strand break repair (17) and base-excision repair (44).

Pol ε purified from *S. cerevisiae* consists of at least four subunits, including the 256-kDa catalytic subunit encoded by *POL2* and three additional subunits of approximately 80, 34, and 29 kDa encoded by *DPB2*, *DPB3*, and *DPB4*, respectively (4, 5, 7, 15). In human cells, Pol ε is also composed of at least four subunits, all of which display significant homology to their yeast counterparts. These include the large catalytic subunit encoded by *POLE* (21), the second-largest subunit homologous to *DPB2*, called *DPE2* (23), and two smaller subunits of approximately 17 and 12 kDa which share homology with *DPB4* and *DPB3*, respectively (24).

Other proteins that interact with and might regulate Pol ε activity include Dpb11p, a multicopy suppressor of both *pol2* and *dpb2* temperature-sensitive mutants (6), and Drc1p (Sld2p), which physically interacts with Dpb11p (19, 43). Dpb11p is required for normal S-phase progression (6, 27) and interacts genetically with Cdc45p, a protein implicated in the assembly of the initiation complex (35, 46). *DPB11* shares homology with $cut5^+$, a gene required for DNA replication initiation and G_2-M checkpoint control in fission yeast (29, 37, 38, 41). However, it is not known whether Cut5p interacts directly with Pol ε or other components of the replicative complex.

Recently, it has been reported that the C-terminal half of Pol ε lacking the conserved polymerase or exonuclease domains is sufficient to rescue a *pol2* null mutant in *S. cerevisiae* (12, 22). Surprisingly, these cells displayed only marginal defects in either DNA replication or DNA repair and did not require the checkpoint gene *MEC1* for viability (12, 22). Here we demonstrate that *Schizosaccharomyces pombe* cells lacking the N terminus of Pol ε ($cdc20^{\Delta N-term}$) are also viable. However, these cells display increased sensitivity to DNA-damaging agents and have a cell cycle delay. Moreover, cell viability is dependent on the DNA damage checkpoint control. These data demonstrate that the C terminus of Pol ε has a critical role in DNA replication that does not rely on its ability to synthesize DNA. Considering that these two yeasts are evolutionarily distant, our results suggest that the N terminus of Pol ε may be dispensable in all eukaryotic cells. Based on our earlier observation that *cdc20* temperature-sensitive mutants show cell cy-

---

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, P.O. Box 016129, University of Miami School of Medicine, Miami, FL 33101-6129. Phone: (305) 243-3105. Fax: (305) 243-3064. E-mail: gdurso@miami.edu.

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

Strain or mutation	Genotype	Source
972	h^-	P. Nurse
cdc20-M10	h^- cdc20-M10 leu1-32 ura4-D18	P. Nurse
cdc20-P7	h^- cdc20-P7 leu1-32	P. Nurse
Δcdc20/cdc20⁺	h^-/h^+ cdc20⁺/cdc20::ura4⁺ ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18	P. Nurse
cdc20^ΔN-term	h^- cdc20::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20C1 int pRep6X	This study
cdc20^N-term+C-term	h^- cdc20::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20C1 int pRep6X-cdc20N	This study
cdc20^3hacdc20+	h^- cdc20::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20⁺	This study
cdc20^tsN-term+C-term	h^- cdc20::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20C1 int pRep6X-cdc20N(M10)	This study
Δhus1	h^- hus1::leu2⁺ ade6-704 leu1-32 ura4-D18	T. Enoch
Δrad3	h^- rad3::ura4⁺ ade6-704 leu1-32 ura4-D18	A. Carr
Δcds1	h^- cds1::ura4⁺ ade6-704 leu1-32 ura4-D18	P. Nurse
Δchk1	h^- chk1::ura4⁺ ade6-704 leu1-32 ura4-D18	P. Nurse
cdc20^N-term+C-term Δchk1	h^+ cdc20::ura4⁺ chk1::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20C1 int pRep6X-cdc20N	This study
cdc20^tsN-term+C-term Δchk1	h^+ cdc20::ura4⁺ chk1::ura4⁺ ade6-704 leu1-32 ura4-D18 int pJK148-nmt41-3hacdc20C1 int pRep6X-cdc20N-M10	This study

cle arrest early in S phase, we propose that the function of the C terminus of Pol ε is to ensure proper assembly of the DNA replicative complex.

MATERIALS AND METHODS

Yeast strains and methods. All fission yeast strains used for this study were derived from *S. pombe* strains 972 and 975 and are listed in Table 1. All media, growth conditions, and genetic manipulations were used as previously described (31).

Molecular cloning and the construction of *cdc20^ΔN-term*, *cdc20^N-term+C-term*, and *cdc20^3hacdc20+* strains. The sequence corresponding to the C terminus of the product of *cdc20⁺* was amplified by PCR using the forward primer 5′GGAAT TCCATATGCGTCTAGGATCAGTAGTAC3′ and the reverse primer 5′CCC CCCGGGGGGCATGAGTGGAAAAATGG3′, tagged with *Nde*I and *Sma*I as underlined. The resulting 3.4-kb fragment encoding the C terminus of Pol ε (amino acids 1141 to 2199) was cloned into pRep1, generating pRep1-*cdc20C1*. Further truncations of *cdc20C1* yielded *cdc20C2* and *cdc20C3*. To generate pRep1-*nlscdc20C3*, a PCR fragment containing the putative nuclear localization signal (NLS) was amplified and cloned into pRep1-*cdc20C3* at the *Nde*I and *Bam*HI sites.

To construct the *cdc20^ΔN-term* strain, the *cdc20C1* gene was first cloned into pARC613, tagging the gene with three tandem copies of the hemagglutinin (HA) epitope. A 5.6-kb *Pst*I/*Sac*I fragment from pARC613-*cdc20C1* was then cloned into pJK148 (20). The plasmid pJK148-*cdc20C1* was linearized at the *Bsu*36I site within the *leu1⁺* gene and transformed into the Δcdc20/cdc20⁺ diploid strain (*cdc20⁺/cdc20::ura4⁺ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 h⁺/h⁻*). Stable integrants were isolated and induced to sporulate under low-nitrogen conditions. Spores were then germinated on minimal medium lacking uracil and leucine. The *ade6-M210* marker was then removed by backcrossing to *leu1-32 ura4-D18* and selecting for leucine and uracil prototrophs, yielding *cdc20^ΔN-term*. Integration of the *cdc20C1* gene at the *leu1* site was confirmed by Southern blot hybridization (see Fig. 3B).

To create the *cdc20^N-term+C-term* strain, the sequence corresponding to the N terminus of the *cdc20⁺* product (from amino acid 1 to 1281) was amplified using the forward primer 5′CGGCGGTCGACTATGCCCTTAAAAACAGCTCG3′ and reverse primer 5′GCCGAACCCGGGGAATTGCCTTGATTGAAACC3′, tagged with *Sal*I and *Sma*I, respectively. The 3.8-kb fragment was cloned into pRep6X that contains the *sup3-5* allele, a suppressor of the *ade6-704* mutant allele, thus creating pRep6X-*cdc20N*. This plasmid was transformed into a *cdc20^ΔN-term ade6-704* mutant. Stable integrants were selected on minimal medium containing a low level of adenine. The *cdc20^tsN-term+C-term* strain was generated in a similar manner, except that the sequence corresponding to the N terminus of the product of *cdc20⁺* was PCR amplified from genomic DNA derived from *cdc20-M10*.

To generate the control (*cdc20^3hacdc20+*) strain, the sequence corresponding to the N terminus of the *cdc20⁺* product was amplified by PCR using the forward primer 5′CGGCGGAGATCTATGCCCTTAAAAACAGCTCG3′ (tagged with *Bgl*II as underlined) and the reverse primer 5′CGATTTCATCAACATTGACG3′. The 1.7-kb PCR product was digested with *Bgl*II and *Bam*HI and cloned into the *Bam*HI site of pARC613. The 2.9-kb *Pst*I/*Sma*I fragment from the resulting plasmid, pARC613-*cdc20N*, was then cloned into pJK148. This plasmid was digested with *Apa*I and *Sma*I and ligated to a 4.0-kb *Apa*I/*Pst*I fragment and a 1.8-kb *Pst*I/*Sma*I fragment from pIRT2-*cdc20⁺* and pRep1-*cdc20C1* plasmids, respectively, in a three-way ligation. The resulting plasmid, pJK148-*3hacdc20⁺*, was linearized with *Bsu*36I in the *leu1⁺* marker and transformed into the Δcdc20 strain. All additional steps are identical to those used for the generation of *cdc20^ΔN-term*.

Determination of cell generation time. Cells were grown for at least eight generations in minimal medium at 32°C prior to analysis. Samples were collected every hour and counted using a hemacytometer. The cell generation time, *T*, was calculated as the log ($2^{t2−t1}$)/log (*y/x*), where *y* is the number of cells/ml at time t_2 and *x* is the number of cells/ml at time t_1.

Cell synchronization using *cdc10-129*. To block cells in pre-Start G₁ phase using the *cdc10-129* mutation (34), cells were incubated in minimal media at 36°C for 4 h. Cells were released from the G₁ block by rapidly cooling cultures to 25°C. Samples were collected every 15 minutes and fixed in 70% ethanol for fluorescence-activated cell sorter (FACS) analysis and microscopic examination.

Flow cytometry analysis and microscopic examination. For DNA content measurements, cells were stained with propidium iodide and analyzed by FACS as described previously (31). For microscopic examination, cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) and examined with a Zeiss fluorescence microscope.

Cell survival rate measurements. Cells were grown to mid-log phase (optical density at 595 nm, 0.3 to 0.5) in minimal media prior to treatment with either hydroxyurea (HU) (12 mM) or methylmethane sulfonate (MMS) (0.2%). Cell samples were collected, diluted, plated on minimal medium, and incubated for 4 days at 32°C. The number of colonies was determined and plotted as the percent viability relative to the untreated control. For the UV sensitivity assay, cells were irradiated with increasing doses of UV light (254 nm) in a GS Gene Linker (Bio-Rad, Hercules, Calif.). Total output energy (in millijoules) was measured by an internally mounted photodetector. The gene linker was programmed to release a specific amount of total energy from 1 to 5 mJ in 1-mJ increments. Following irradiation, equal numbers of cells (approximately 500) were plated on minimal agar plates and incubated for 4 days at 32°C. The number of colonies was determined, and data were analyzed using SigmaPlot software.

RESULTS

Expression of the C-terminal half of Pol ε complements *cdc20^ts* mutants. To determine if the C-terminal half of Pol ε can complement *cdc20* mutants, we transformed two different alleles, *cdc20-M10* and *cdc20-P7*, with the plasmid pRep1-*cdc20C1* (Fig. 1). This plasmid contains a gene encoding the C terminus of Pol ε from amino acid 1141 to 2199 expressed under the control of the thiamine-repressible *nmt1* promoter (28). This deletion removes all the conserved polymerase and exonuclease domains of Pol ε. Transformed colonies were selected at the permissive temperature of 25°C and then streaked out on minimal agar at both 25°C and the nonpermissive tem-
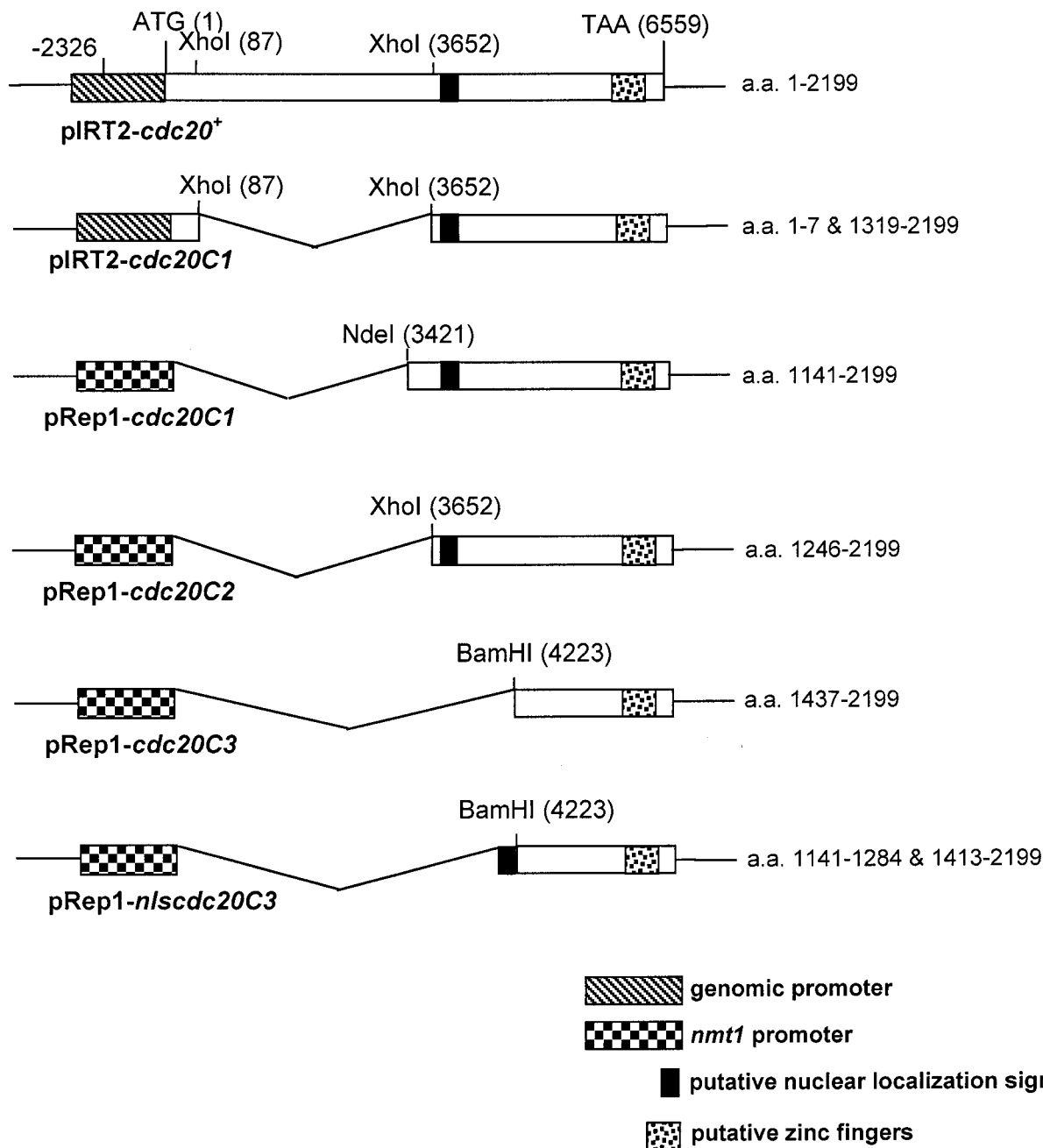
FIG. 1. Schematic representation of N-terminally truncated forms of *cdc20* that were used for the complementation analysis shown in Fig. 2 (the gene structure is not drawn in scale). All plasmids were derived from pIRT2-*cdc20*⁺, which contains a 10,054-bp genomic fragment of the *cdc20*⁺ gene. The numbers in parentheses indicate nucleotide positions. The first nucleotide of the start codon was numbered 1, and all other sequences were designated accordingly. The numbers on the right of each plasmid indicate the numbers of amino acids of Pol ε being included in each construct. The series of pRep1 plasmids with which the C-terminal fragments of the *cdc20* product were expressed under the *nmt1* promoter were generated by cloning restriction fragments of pIRT2-*cdc20*⁺ into the pRep1 vector. Initiation of translation is presumed to take place at the first internal methionine. The putative NLS and zinc finger motifs are as indicated.

perature of 36°C. Both *cdc20-M10* and *cdc20-P7* transformed with pRep1-*cdc20C1* but not with pRep1 alone were able to grow at the restrictive temperature, demonstrating that expression of the C terminus of Pol ε can rescue the temperature sensitivity of the *cdc20* mutants (Fig. 2A).

To identify the minimal C-terminal sequences required for complementation, we transformed the *cdc20* mutant strains with plasmids containing further truncations of the *cdc20C1* gene. Since we previously demonstrated that the extreme C terminus of *cdc20*⁺ is essential for cell viability (14), we deleted sequences from the N-terminal end only. Two additional plasmids were generated, pRep1-*cdc20C2* and pRep1-*cdc20C3*,
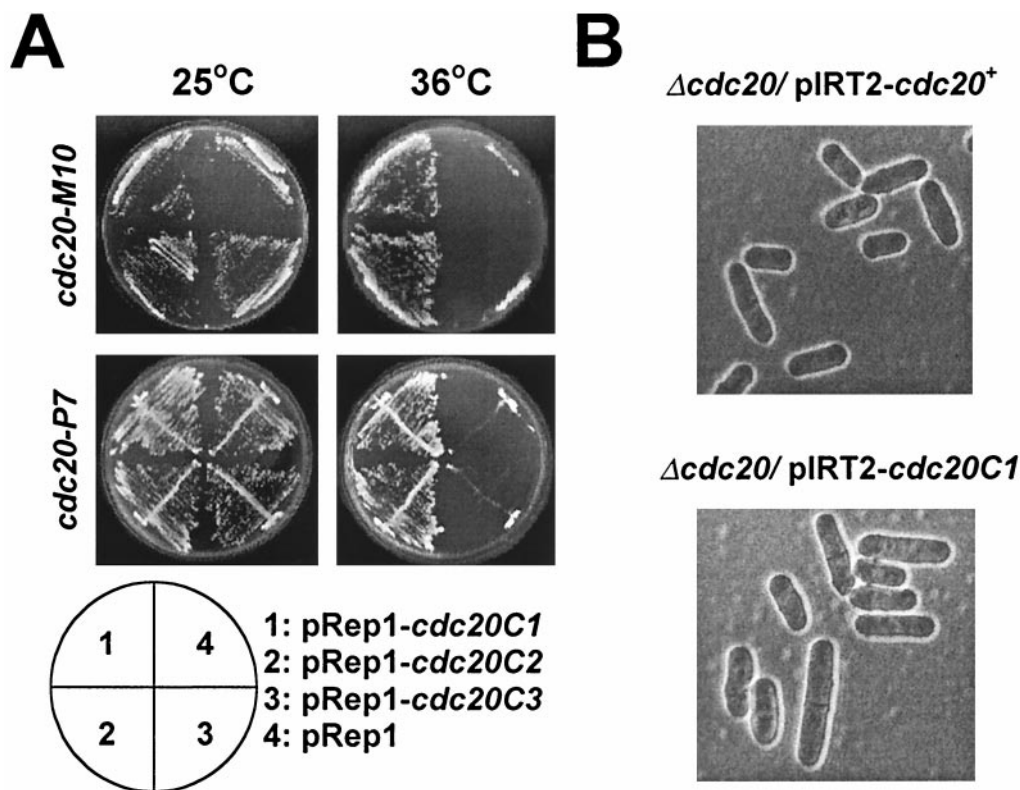
FIG. 2. Expression of the C-terminal half of Pol ε rescues both $cdc20^{ts}$ mutants and cells with the complete $cdc20^+$ gene deleted. (A) Complementation of the temperature-sensitive $cdc20$-M10 (top) and $cdc20$-P7 (bottom) strains by transformation with plasmids expressing the C-terminally truncated forms of Pol ε. Transformants were streaked on minimal agar and incubated at 25°C (left) and 36°C (right). (B) Expression of the C-terminal half of the $cdc20$ product can rescue the Δ$cdc20$ strain. The $cdc20^+$/Δ$cdc20$ diploid strain was transformed with pIRT2-$cdc20^+$ or pIRT2-$cdc20C1$. Following sporulation and germination of positive transformants, haploid cells containing the deletion of $cdc20^+$ and either the plasmid pIRT2-$cdc20^+$ (top) or pIRT2-$cdc20C1$ (bottom) were selected and visualized by phase contrast microscopy.

encoding Pol ε from amino acid 1246 to 2199 and from amino acid 1437 to 2199, respectively (Fig. 1). We observed that pRep1-$cdc20C2$ but not pRep1-$cdc20C3$ was able to complement both $cdc20$-M10 and $cdc20$-P7 (Fig. 2A). Sequence analysis using ProfileScan revealed a putative bipartite NLS located at amino acids 1257 to 1274. To test whether the inability of pRep1-$cdc20C3$ to rescue the $cdc20$ mutants was due to deletion of the NLS, this sequence was fused to $cdc20C3$ to create the plasmid pRep1-$nlscdc20C3$ (Fig. 1). However, expression of this fusion protein failed to rescue either $cdc20$-M10 or $cdc20$-P7 (data not shown). We conclude that the minimal Pol ε sequences required for complementation of the $cdc20$ mutants include amino acids 1246 to 2199.

Expression of the C-terminal half of Pol ε rescues a deletion of the $cdc20^+$ gene. Our observation that the C-terminal half of Pol ε is capable of rescuing $cdc20$ mutants was surprising considering that the mutations in both the $cdc20$-M10 and $cdc20$-P7 strains map to the N-terminal half of the protein (unpublished observations). Therefore, we tested whether expression of the C terminus of Pol ε alone can rescue a strain with the entire $cdc20^+$ gene deleted. We transformed pRep1-$cdc20C1$ into the Δ$cdc20$/$cdc20^+$ diploid strain, in which a single copy of $cdc20^+$ has been replaced by $ura4^+$ (14). In addition, the Δ$cdc20$/$cdc20^+$ strain was transformed with pIRT2-$cdc20^+$ and pIRT2-$cdc20C1$, expressing either the

$cdc20^+$ gene or $cdc20$-C1 under the control of the endogenous $cdc20$ promoter. Diploids transformed with these plasmids were induced to sporulate, and haploid cells prototrophic for uracil and leucine were selected. Cell growth was observed only following transformation with pRep1-$cdc20C1$, pIRT2-$cdc20^+$, and pIRT2-$cdc20C1$ (Fig. 2B, cells transformed with pIRT2-$cdc20^+$ and pIRT2-$cdc20C1$) but not with the vector alone or with a nonrelevant gene. Consistent with our earlier results, $cdc20C2$, but not $cdc20C3$, was able to rescue the deletion of $cdc20^+$. We then generated a $cdc20^{\Delta N\text{-}term}$ strain, in which $cdc20^+$ is deleted but which contains an integrated copy of $3hacdc20C1$ under the control of the $nmt41$ promoter. Southern blot analysis confirmed the absence of wild-type $cdc20^+$ and the presence of $cdc20C1$ near the $leu1$ locus in this strain (Fig. 3A and B). Western blot analysis of cellular extracts prepared from the $cdc20^{\Delta N\text{-}term}$ strain identified a polypeptide with a molecular weight consistent with that of 3HACdc20C1p that was not present in extracts prepared from wild-type cells (Fig. 3C).

The $cdc20^{\Delta N\text{-}term}$ strain shows a delay in cell cycle progression and is sensitive to DNA-damaging agents. So far, we have shown that the N-terminal catalytic domains of Pol ε are dispensable for cell viability in fission yeast. To address whether the absence of the N terminus of Pol ε has any effect on DNA replication or DNA repair, we tested whether the $cdc20^{\Delta N\text{-}term}$
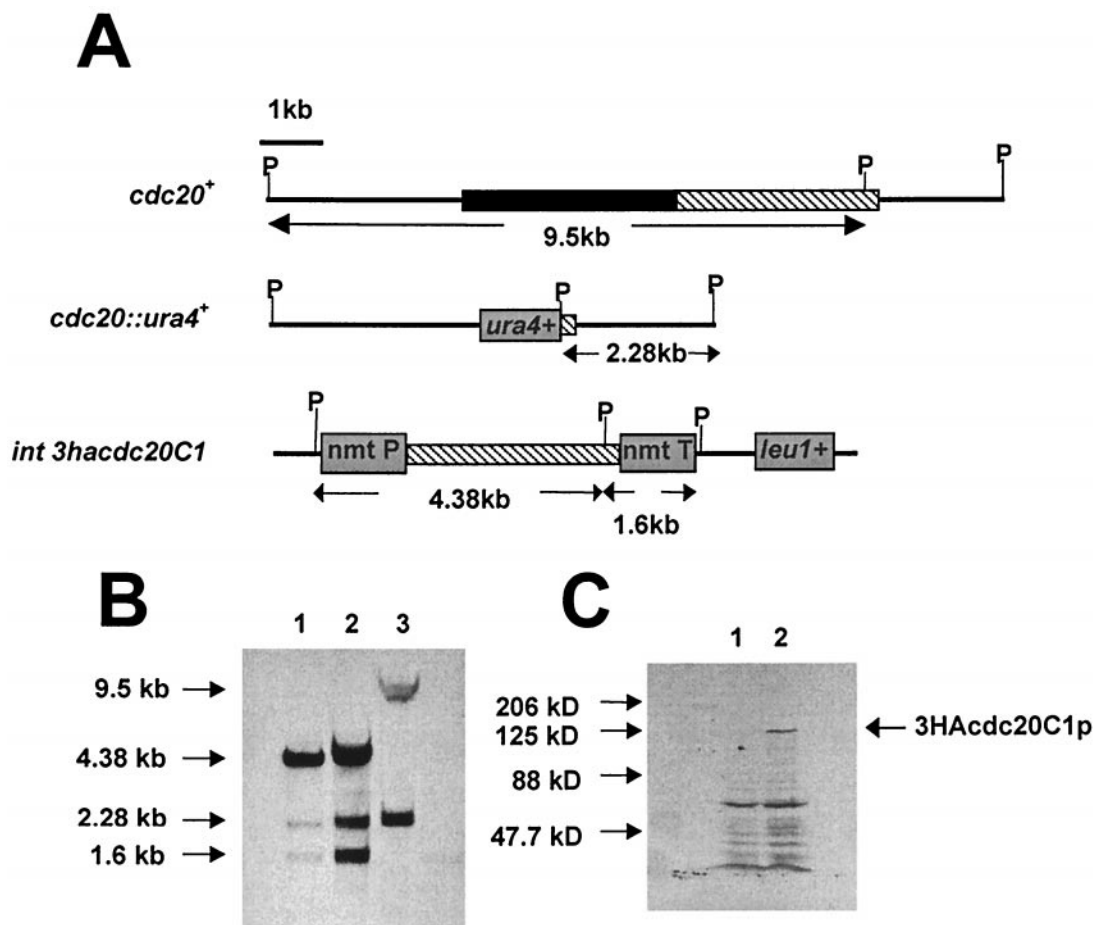
FIG. 3. Construction of the $cdc20^{\Delta N\text{-}term}$ mutant. (A) Expected genomic structure of the $cdc20$ and $leu1$ loci following integration of pJK148-$cdc20C1$. The solid and cross-hatched bars indicate the regions of $cdc20$ corresponding to the N terminus and the C terminus, respectively. P indicates the location of the $PstI$ restriction sites used for the Southern blot analysis. (B) Southern blot of genomic DNA prepared from the diploid $\Delta cdc20/cdc20^+$ strain (lane 3) and from two independent $cdc20^{\Delta N\text{-}term}$ isolates (lanes 1 and 2), probed with a PCR fragment corresponding to the C-terminal half encoded by $cdc20$. (C) Western blot of a protein extract prepared from wild-type cells (lane 1) and $cdc20^{\Delta N\text{-}term}$ cells (lane 2), using anti-HA monoclonal antibodies. The apparent molecular mass of 3HACdc20C1p is approximately 122 kDa.

strain is sensitive to either replication blocks or DNA-damaging agents. In these experiments, cell viability was monitored following treatment of the $cdc20^{\Delta N\text{-}term}$ strain with HU, MMS, and UV irradiation. Although our results demonstrate that the $cdc20^{\Delta N\text{-}term}$ strain displays normal sensitivity to HU (Fig. 4A), these cells show increased sensitivity to both UV irradiation and MMS (Fig. 4B and C). These data suggest that cells lacking the N-terminal domains of Pol ε are defective in DNA repair.

To test whether expression of the N terminus of Pol ε in *trans* can complement the defects in $cdc20^{\Delta N\text{-}term}$ cells, we generated a plasmid Rep6X-$cdc20N$ expressing the N-terminal half of Pol ε (from amino acid 1 to 1281). This clone was then integrated into the $cdc20^{\Delta N\text{-}term}$ strain, generating the $cdc20^{N\text{-}term+C\text{-}term}$ strain. Thus, in $cdc20^{N\text{-}term+C\text{-}term}$ cells, the N- and C-terminal domains of Pol ε are expressed from two independent genes. First, we measured the cell generation time for each strain; the results are summarized in Table 2. As expected, the $cdc20^{3hacdc20^+}$ control strain has a generation time of approximately 2.3 h in minimal medium at 32°C, identical to that of wild-type (972) cells. In contrast, the $cdc20^{\Delta N\text{-}term}$

strain is delayed approximately 80 min. Interestingly, the generation time of the $cdc20^{N\text{-}term+C\text{-}term}$ strain is similar to that of the wild type, suggesting that expression of the N terminus in *trans* can rescue the slow-growth phenotype. We then measured the survival rate of each strain after exposure to HU and DNA-damaging agents. As mentioned above, $cdc20^{\Delta N\text{-}term}$ cells are resistant to HU but are sensitive to both UV irradiation and MMS. Interestingly, expression of the N-terminal half of Pol ε in $cdc20^{\Delta N\text{-}term}$ cells is able to restore the survival rate after exposure to UV and MMS to levels comparable to those for wild-type cells (Fig. 4B and C). These results suggest that not only is the N terminus of Pol ε important for DNA repair, but it can still function when physically separated from the C-terminal half of the enzyme.

$cdc20^{\Delta N\text{-}term}$ **cells are delayed during S phase.** To determine if the longer generation time of the $cdc20^{\Delta N\text{-}term}$ strain is due to a delay during specific phases of the cell cycle, we monitored the timing of both S phase and mitosis by monitoring DNA content and the appearance of binucleate cells following release from a G_1 block. To do this, we constructed the $cdc10$-$129\ cdc20^{\Delta N\text{-}term}$ double mutant and shifted these cells to the
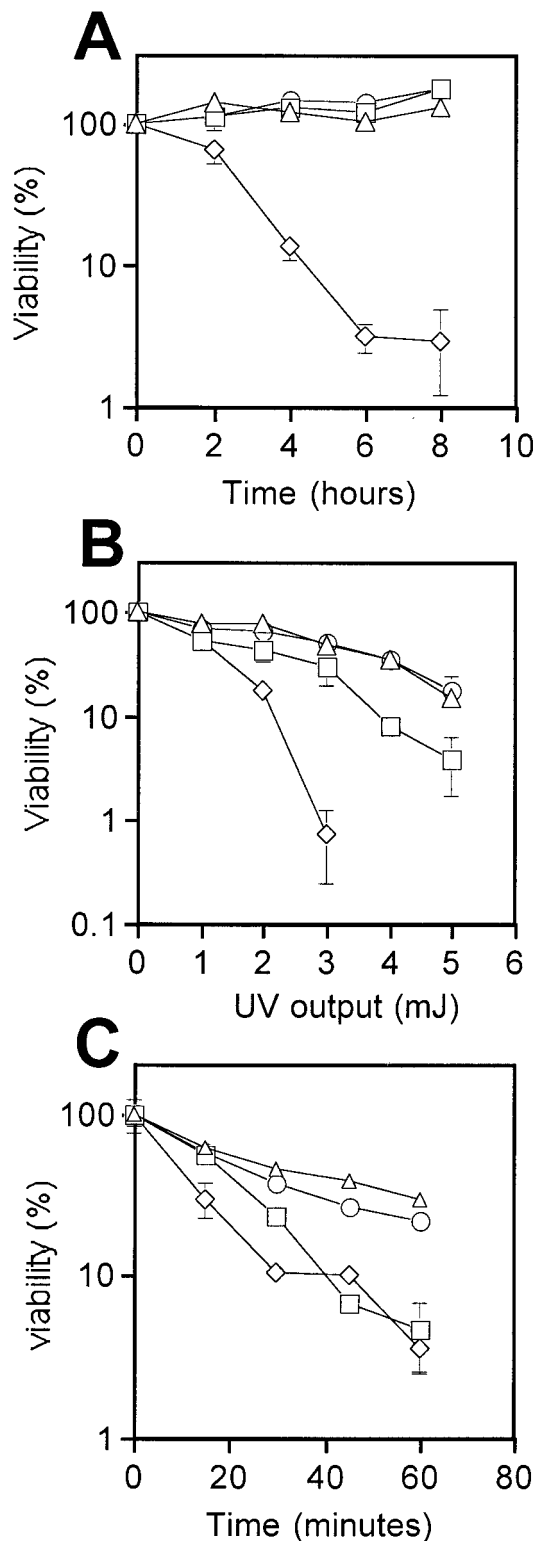
FIG. 4. Cells lacking the N-terminal half of Pol ε display increased sensitivity to DNA damage. Survival rates of $cdc20^{3hacdc20+}$ (triangle), $cdc20^{\Delta N\text{-}term}$ (square), $cdc20^{N\text{-}term+C\text{-}term}$ (circle), $hus1\text{-}14$ (diamond in panel A), and $rad2\text{-}44$ (diamonds in panels B and C) cells. Following treatment with 11 mM HU (A), increasing doses of UV irradiation (B), and 0.2% MMS (C), cells were plated at 32°C for 3 days, colonies were counted, and the survival rate was determined by SigmaPlot. Error bars indicate standard deviations.

TABLE 2. Summary of physiological characterizations of $cdc20$ mutants

Strain or mutation	Generation time (h)[b]	Resistance[c] to:		
		HU	UV	MMS
972 (WT)[a]	2.32	+++	+++	+++
$cdc20^{3hacdc20+}$	2.30	+++	+++	+++
$cdc20^{\Delta N\text{-}term}$	3.63	+++	++	+
$cdc20^{N\text{-}term+C\text{-}term}$	2.36	+++	+++	++

[a] WT, wild type.
[b] Generation time was measured in minimal medium at 32°C.
[c] +, poor resistance, +++, normal resistance.

restrictive temperature for $cdc10\text{-}129$ (36°C), causing cell cycle arrest in G_1. Upon return to the permissive temperature of 25°C, cells enter S phase synchronously (Fig. 5A). In three independent experiments, we observed that mitosis is delayed approximately 1 h in $cdc20^{\Delta N\text{-}term}$ cells compared to results with either $cdc20^{3hacdc20+}$ or $cdc20^{N\text{-}term+C\text{-}term}$ cells (Fig. 5B). Analysis of DNA content by FACS shows that S phase is approximately 30 to 60 min slower in the $cdc20^{\Delta N\text{-}term}$ strain compared to results with cells containing an intact Pol ε (Fig. 5A, compare $cdc20^{\Delta N\text{-}term}$, 90 min, to $cdc20^{3hacdc20+}$, 90 min). It is not known whether this delay reflects inefficient DNA replication initiation or elongation. However, unlike when DNA replication is inhibited by treatment with HU, $cdc20^{\Delta N\text{-}term}$ cells do not require the checkpoint gene $cds1^+$ for viability (see below) (Table 3). Consistent with the results of the DNA damage sensitivity assays and the cell cycle generation time measurements, expression of the N terminus in *trans* was able to rescue the S phase delay (Fig. 5, see results for $cdc20^{N\text{-}term+C\text{-}term}$). These experiments suggest that cells lacking the N terminus of Pol ε undergo a defective round of DNA synthesis. To test the possibility that DNA damage accumulates in these cells and contributes to the cell cycle delay, we examined whether the DNA damage checkpoint is required for cell viability in the $cdc20^{\Delta N\text{-}term}$ strain.

The viability of the $cdc20^{\Delta N\text{-}term}$ mutant is dependent on the DNA damage checkpoint control. Our data have shown that S phase is delayed in the $cdc20^{\Delta N\text{-}term}$ strain. Eukaryotic cells respond to DNA replication blocks or DNA damage by activating checkpoint controls that delay the onset of mitosis until DNA replication and repair are completed (2, 13, 16, 33, 45). A number of *S. pombe* genes have been identified that are essential to activate the checkpoint control. These include the *rad* genes (*rad* 1, 3, 9, 17, and 26), *hus1*, *chk1*, and *cds1*. The *rad* and *hus* genes are thought to be involved in the recognition of DNA damage and generation of checkpoint signals that ultimately block the onset of mitosis by inhibiting the mitotic kinase Cdc2p (10). Cds1p and Chk1p are two protein kinases that function downstream in the checkpoint control pathway during S and G_2 phases, respectively (1, 25, 32, 36, 42). In addition to its proposed role in the checkpoint control, Cds1p has an additional role during recovery from replication blocks imposed by HU (8, 26). To test whether any of the checkpoint genes are required for viability in $cdc20^{\Delta N\text{-}term}$ cells, we crossed the $cdc20^{\Delta N\text{-}term}$ strain with various checkpoint mutants, including the $\Delta rad3$, $\Delta hus1$, $\Delta chk1$, and $\Delta cds1$ mutants, and the viability of the double mutants was examined by tetrad analysis. We found that *hus1*, *rad3*, and *chk1* are all essential for
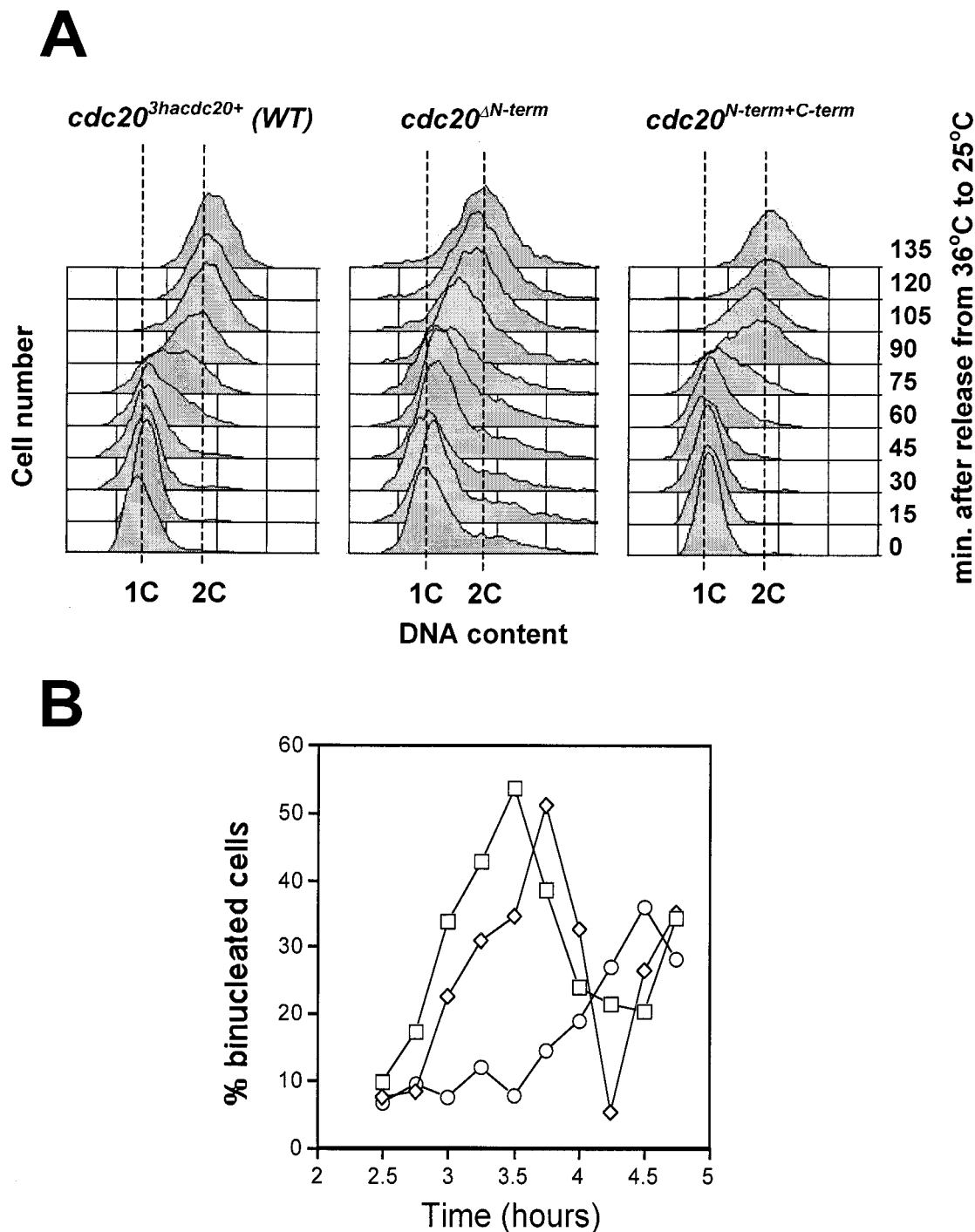
A

B

FIG. 5. Cells lacking the N-terminal half of Pol ε show delayed S and G_2 phases of the cell cycle. (A) FACS analysis of the DNA content of cells released from the *cdc10-129* cell cycle arrest. Samples were collected every 15 min for approximately 2 h. The 1C and 2C DNA control peaks are indicated. (B) Percentage of binucleate cells for *cdc10-129 cdc20³ʰᵃᶜᵈᶜ²⁰⁺* (square), *cdc10-129 cdc20ᐃᴺ⁻ᵗᵉʳᵐ* (circle), and *cdc10-129 cdc20ᴺ⁻ᵗᵉʳᵐ⁺ᶜ⁻ᵗᵉʳᵐ* (diamond) strains at the indicated times following release from the G_1 block.

viability in the *cdc20ᐃᴺ⁻ᵗᵉʳᵐ* strain, demonstrating that *cdc20ᐃᴺ⁻ᵗᵉʳᵐ* requires the DNA damage checkpoint control.

To confirm that the lethality of the *cdc20ᐃᴺ⁻ᵗᵉʳᵐ* cells when combined with DNA damage checkpoint mutations is indeed due to a checkpoint failure, we examined more closely the

terminal phenotype of the *cdc20ᐃᴺ⁻ᵗᵉʳᵐ Δchk1* double mutant. To do this, we first constructed the *cdc20ᵗˢᴺ⁻ᵗᵉʳᵐ⁺ᶜ⁻ᵗᵉʳᵐ* strain, which is identical to the *cdc20ᴺ⁻ᵗᵉʳᵐ⁺ᶜ⁻ᵗᵉʳᵐ* strain except that the N terminus was derived from the temperature-sensitive *cdc20-M10* strain. We then showed that the *cdc20ᵗˢᴺ⁻ᵗᵉʳᵐ⁺ᶜ⁻ᵗᵉʳᵐ*

TABLE 3. Summary of genetic interactions of *cdc20* mutants

Mutation	Viability of cells[a]		
	cdc20[3hacdc20+] (WT)	*cdc20*[ΔN-term]	*cdc20*[N-term+C-term]
Δ*chk1*	+	−	+
Δ*cds1*	+	+	+
Δ*chk1* Δ*cds1*	+	N/A	+
Δ*rad3*	+	−	+
Δ*hus1*	+	−	+
cdc6-23	+	−	+

[a] +, viable; −, synthetically lethal; N/A, not analyzed; WT, wild type.

strain is viable at 36°C, demonstrating that inactivation of the N terminus of Pol ε, when physically separated from the C terminus, does not interfere with normal C-terminal function. However, we found that the *cdc20*[tsN-term+C-term] Δ*chk1* double mutant rapidly lost viability upon a shift to the restrictive temperature (Fig. 6A). Microscopic examination of these cells after staining them with the DNA-binding dye DAPI revealed a high incidence of aberrant mitoses typical of the cut (cell untimely torn) phenotype (Fig. 6C). The appearance of mitotic abnormalities correlated with the observed decrease in cell viability (Fig. 6B). As a control, *cdc20*[N-term+C-term] Δ*chk1* cells displayed no aberrant mitoses at 36°C. These results confirmed that in the absence of the N terminus of Pol ε, cells are dependent on the DNA damage checkpoint control. Considering that Chk1p is required only for the DNA damage checkpoint operating in G$_2$ (1, 41), these data provide evidence that G$_2$ is also delayed in these cells. Interestingly, we found that none of the checkpoint genes are required for viability of *cdc20*[N-term+C-term] cells, providing further support that the N terminus of Pol ε is functional when expressed independently from the C-terminal half of the enzyme.

DISCUSSION

The precise role of Pol ε in eukaryotic DNA replication remains to be resolved. Recently, the N-terminal half of Pol2p, which contains all the conserved catalytic domains of Pol ε, has been shown to be dispensable for cell viability in *S. cerevisiae* (12, 22). This suggests that the essential function of Pol ε does not rely on its ability to synthesize DNA. Consistent with this observation, we demonstrate that fission yeast cells with the N-terminal half of Pol ε deleted are also viable. The fact that these two organisms are evolutionarily distant suggests that this is a conserved feature of Pol ε in all eukaryotic cells.

However, in contrast to *S. cerevisiae*, *S. pombe* cells lacking the N-terminal domains are sensitive to DNA-damaging agents, have a cell cycle delay, and require the DNA damage checkpoint to maintain cell viability. These results suggest that the N terminus, although dispensable for cell viability, normally participates in both DNA replication and repair. Expression of the N-terminal half of Pol ε in *trans* in *cdc20*[ΔN-term] cells rescues the DNA damage sensitivity and alleviates the dependency on the DNA damage checkpoint control, suggesting that the N terminus of Pol ε is active when expressed independently of the C-terminal half of the enzyme.

Analysis of cell cycle progression in *cdc20*[ΔN-term] cells demonstrates that S phase is delayed at least 30 min at 25°C.

Furthermore, cell viability is dependent on expression of the checkpoint genes *rad3*, *hus1*, and *chk1*, suggesting that the cell cycle is also delayed in G$_2$ in response to DNA damage. We have considered two possible models to explain the checkpoint dependency of these cells. In the first model, we propose that in the absence of the N terminus of Pol ε, DNA synthesis occurs inefficiently, as suggested by the slower S phase, and is error prone. Under these conditions, accumulation of excess DNA damage leads to activation of the checkpoint control. DNA damage might result from inefficient chain elongation and subsequent DNA strand breaks or from nucleotide misincorporations due to inefficient proofreading. In this model, the primary function of the N terminus of Pol ε is in DNA replication, with a secondary role in DNA repair. In our second model, DNA damage that normally occurs during S phase is inefficiently repaired in cells lacking the N terminus of Pol ε. This leads to a checkpoint-dependent cell cycle delay. In this model, the primary role of the N terminus is in DNA repair. Currently, it is difficult to distinguish between these two possibilities.

The precise role of the C terminus of Pol ε in DNA replication remains unclear. Based on our results that temperature-sensitive mutants in Pol ε show cell cycle arrest early in S phase (14) and that the N-terminal catalytic domains are not essential for DNA synthesis (this study), we propose that Pol ε, through its C-terminal domain, is required for assembly of the replicative complex. Interestingly, in *S. cerevisiae*, Pol ε is found associated with ARS elements during initiation of DNA replication (3), consistent with our hypothesis that Pol ε functions early in S phase. Moreover, Pol ε was found to associate with replication origins prior to Pol α, a striking result considering that Pol α has been generally believed to be the first polymerase that binds to origins during initiation (27, 30). Whether this reflects a requirement for Pol ε for the assembly of the Pol α-primase complex to DNA is not yet known. In the absence of the N-terminal domain of Pol ε, we predict that the C terminus can interact with other DNA polymerases, such as Pol δ, which can then substitute for the N terminus of Pol ε in DNA synthesis. Consistent with this hypothesis, we have found that *cdc20*[ΔN-term] is synthetically lethal with *cdc6-23*, a temperature-sensitive mutant defective in the catalytic subunit of DNA Pol δ (Table 3).

Analysis of the amino acid sequence of the C-terminal half of Pol ε has not revealed any obvious protein function(s). The most striking feature consists of a series of zinc finger DNA binding motifs at the extreme end of the protein. Site-specific mutagenesis of some of the key cysteine residues within the zinc finger domains has shown that this region of the protein is essential for cell viability in *S. cerevisiae* (11, 12). In *S. pombe*, short C-terminal truncations of Pol ε near the zinc finger motifs are lethal (14). Comparison of the C-terminal sequences of Pol ε from different organisms reveals substantial sequence similarity (>30% identity); however, this is significantly less than what is observed within the N-terminal catalytic domain (>60% identity). This suggests that the polymerase function of Pol ε is much less tolerant of genetic change than is the C-terminal domain, which has clearly undergone considerable species-dependent modifications throughout evolution. It has been reported that *S. pombe* Pol ε is unable to complement mutations in POL2 in *S. cerevisiae* (40). This is likely to reflect
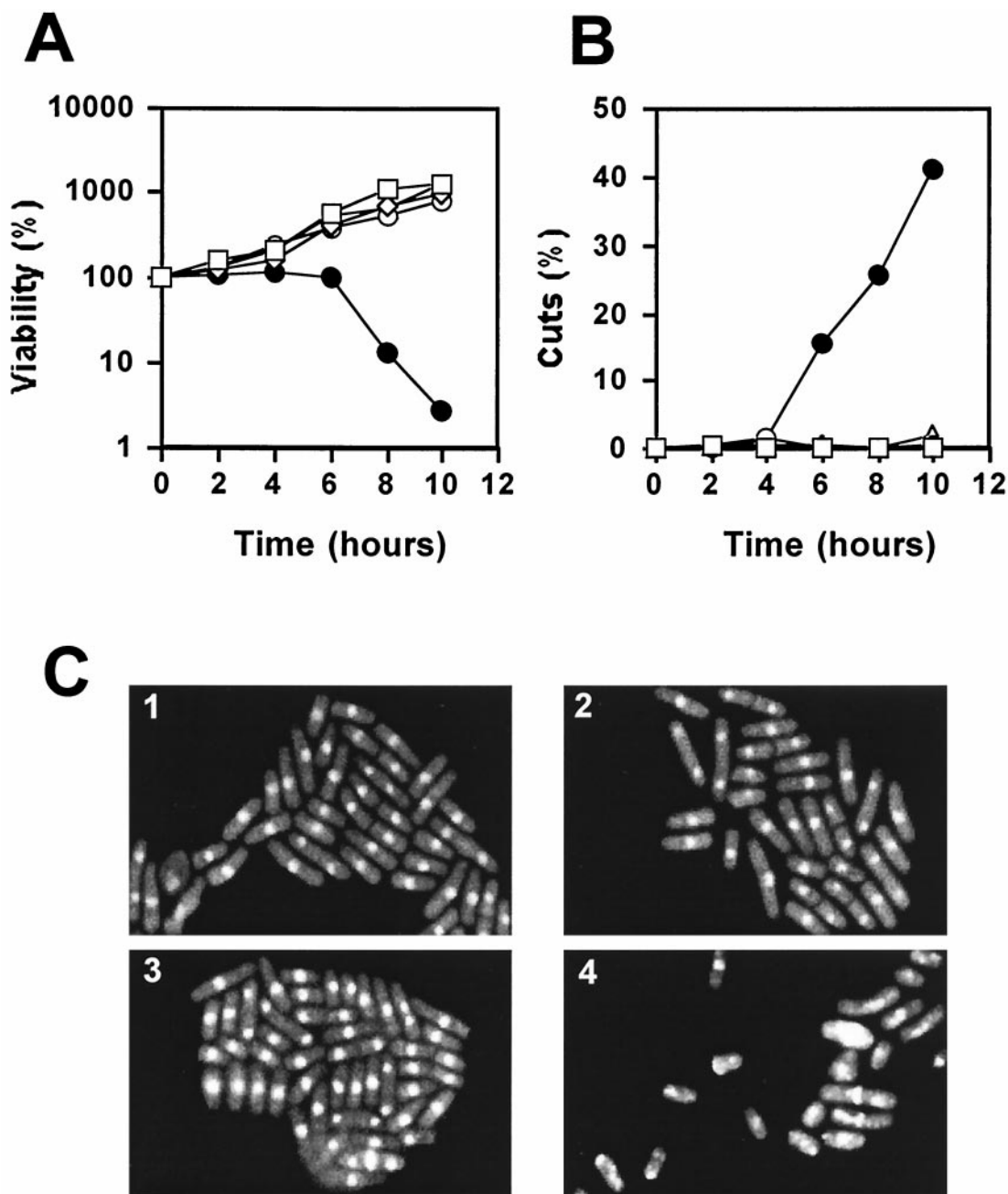
FIG. 6. Cell viability for the *cdc20*$^{\Delta N\text{-}term}$ strain is dependent on the DNA damage checkpoint control. The *cdc20*$^{tsN\text{-}term+C\text{-}term}$ Δ*chk1* double mutant is inviable at 36°C. (A) Exponentially growing cultures of *972*$^+$ (square), *cdc20*$^{N\text{-}term+C\text{-}term}$ (diamond), *cdc20*$^{tsN\text{-}term+C\text{-}term}$ (circle), *cdc20*$^{N\text{-}term+C\text{-}term}$ Δ*chk1* (triangle), and *cdc20*$^{tsN\text{-}term+C\text{-}term}$ Δ*chk1* (closed circle) strains were shifted from 25 to 36°C for 10 h. Samples were collected every hour and plated to determine cell viability. (B) The loss of viability of *cdc20*$^{tsN\text{-}term+C\text{-}term}$Δ*chk1* cells at 36°C correlates with an increase in the number of cells undergoing an aberrant mitosis. These events are plotted as the percentage of "cut" cells and cells with abnormal nuclear morphology. (C) Visualization of the cut phenotype by DAPI staining of nuclei. Panels 1 and 2, *cdc20*$^{N\text{-}term+C\text{-}term}$ Δ*chk1* cells at 25 and 36°C, respectively. Panels 3 and 4, *cdc20*$^{tsN\text{-}term+C\text{-}term}$ Δ*chk1* cells at 25 and 36°C, respectively.

differences within the C-terminal domain. It will be interesting to determine if the N terminus of *S. cerevisiae* or human Pol ε can complement the defects in the *S. pombe cdc20*$^{\Delta N\text{-}term}$ strain. We suspect that the genetic diversity observed within the C terminus reflects species-specific protein-protein interactions that are critical during the early stages of initiation of DNA replication.

Our studies clearly demonstrate that the role of Pol ε in eukaryotic DNA replication is more complex than previously anticipated. Along with its polymerase and exonuclease activities, Pol ε has an additional essential function(s) that resides in the C-terminal half of the enzyme. Future experiments will be focused on providing a better understanding of the structure and function of Pol ε, particularly within the C-terminal domain, and how this important enzyme interacts with other components of the replication machinery.

ACKNOWLEDGMENTS

REFERENCES

1. **al-Khodairy, F., E. Fotou, K. S. Sheldrick, D. J. Griffiths, A. R. Lehmann, and A. M. Carr.** 1994. Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. Mol. Biol. Cell **5:**147–160.

2. **al-Khodairy, F., and A. M. Carr.** 1992. DNA repair mutants defining G2 checkpoint pathways in Schizosaccharomyces pombe. EMBO J. **11:**1343–1350.

3. **Aparicio, O. M., D. M. Weinstein, and S. P. Bell.** 1997. Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell **91:**59–69.

4. **Araki, H., R. K. Hamatake, L. H. Johnston, and A. Sugino.** 1991. DPB2, the gene encoding DNA polymerase II subunit B, is required for chromosome replication in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **88:**4601–4605.

5. **Araki, H., R. K. Hamatake, A. Morrison, A. L. Johnson, L. H. Johnston, and A. Sugino.** 1991. Cloning DPB3, the gene encoding the third subunit of DNA polymerase II of Saccharomyces cerevisiae. Nucleic Acids Res. **19:**4867–4872.

6. **Araki, H., S. H. Leem, A. Phongdara, and A. Sugino.** 1995. Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc. Natl. Acad. Sci. USA **92:**11791–11795.

7. **Araki, H., P. A. Ropp, A. L. Johnson, L. H. Johnston, A. Morrison, and A. Sugino.** 1992. DNA polymerase II, the probable homolog of mammalian DNA polymerase epsilon, replicates chromosomal DNA in the yeast Saccharomyces cerevisiae. EMBO J. **11:**733–740.

8. **Boddy, M. N., B. Furnari, O. Mondesert, and P. Russell.** 1998. Replication checkpoint enforced by kinases Cds1 and Chk1. Science **280:**909–912.

9. **Budd, M. E., and J. L. Campbell.** 1993. DNA polymerases delta and epsilon are required for chromosomal replication in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**496–505.

10. **Caspari, T., and A. M. Carr.** 1999. DNA structure checkpoint pathways in Schizosaccharomyces pombe. Biochimie **81:**173–181.

11. **Dua, R., D. L. Levy, and J. L. Campbell.** 1998. Role of the putative zinc finger domain of Saccharomyces cerevisiae DNA polymerase ε in DNA replication and the S/M checkpoint pathway. J. Biol. Chem. **273:**30046–30055.

12. **Dua, R., D. L. Levy, and J. L. Campbell.** 1999. Analysis of the essential functions of the C-terminal protein/protein interaction domain of Saccharomyces cerevisiae pol epsilon and its unexpected ability to support growth in the absence of the DNA polymerase domain. J. Biol. Chem. **274:**22283–22288.

13. **D'Urso, G., and P. Nurse.** 1995. Checkpoints in the cell cycle of fission yeast. Curr. Opin. Genet. Dev. **5:**12–16.

14. **D'Urso, G., and P. Nurse.** 1997. Schizosaccharomyces pombe cdc20$^+$ encodes DNA polymerase epsilon and is required for chromosomal replication but not for the S phase checkpoint. Proc. Natl. Acad. Sci. USA **94:**12491–12496.

15. **Hamatake, R. K., H. Hasegawa, A. B. Clark, K. Bebenek, T. A. Kunkel, and A. Sugino.** 1990. Purification and characterization of DNA polymerase II from the yeast Saccharomyces cerevisiae. Identification of the catalytic core and a possible holoenzyme form of the enzyme. J. Biol. Chem. **265:**4072–4083.

16. **Hartwell, L., and T. Weinert.** 1989. Checkpoints: controls that ensure the order of cell cycle events. Science **246:**629–634.

17. **Holmes, A. M., and J. E. Haber.** 1999. Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases. Cell **96:**415–424.

18. **Jessberger, R., V. Podust, U. Hubscher, and P. Berg.** 1993. A mammalian protein complex that repairs double-strand breaks and deletions by recombination. J. Biol. Chem. **268:**15070–15079.

19. **Kamimura, Y., H. Masumoto, A. Sugino, and H. Araki.** 1998. Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication. Mol. Cell. Biol. **18:**6102–6109.

20. **Keeney, J. B., and J. D. Boeke.** 1994. Efficient targeted integration at leu1-32 and ura4-294 in Schizosaccharomyces pombe. Genetics **136:**849–856.

21. **Kesti, T., H. Frantti, and J. E. Syvaoja.** 1993. Molecular cloning of the cDNA

22. **Kesti, T., K. Flick, S. Keranen, J. E. Syvaoja, and C. Wittenberg.** 1999. DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. Mol. Cell **3:**679–685.

23. **Li, Y., H. Asahara, V. S. Patel, S. Zhou, and S. Linn.** 1997. Purification, cDNA cloning, and gene mapping of the small subunit of human DNA polymerase epsilon. J. Biol. Chem. **272:**32337–32344.

24. **Li, Y, Z. F. Pursell, and S. Linn.** 2000. Identification and cloning of two histone fold motif-containing subunits of HeLa DNA polymerase epsilon. J. Biol. Chem. **275:**23247–23552.

25. **Lindsay, H. D., D. J. Griffiths, R. J. Edwards, P. U. Christensen, J. M. Murray, F. Osman, N. Walworth, and A. M. Carr.** 1998. S-phase-specific activation of Cds1 kinase defines a subpathway of checkpoint response in *Schizosaccharomyces pombe*. Genes Dev. **12:**382–395.

26. **Liu, V. F., D. Bhaumik, and T. S. Wang.** 1999. Mutator phenotype induced by aberrant replication. Mol. Cell. Biol. **19:**1126–1135.

27. **Masumoto, H., A. Sugino, and H. Araki.** 2000. Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol. Cell. Biol. **20:**2809–2817.

28. **Maundrell, K.** 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:**127–130.

29. **McFarlane, R. J., A. M. Carr, and C. Price.** 1997. Characterisation of the Schizosaccharomyces pombe rad4/cut5 mutant phenotypes: dissection of DNA replication and G2 checkpoint control function. Mol. Gen. Genet. **255:**332–340.

30. **Mimura, M., T. Masuda, T. Matsui, and H. Takisawa.** 2000. Central role for Cdc45 in establishing an initiation complex of DNA replication in Xenopus egg extracts. Genes Cells **5:**469–452.

31. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. **194:**795–823.

32. **Morrison, A., H. Araki, A. B. Clark, R. K. Hamatake, and A. Sugino.** 1990. A third essential DNA polymerase in S. cerevisiae. Cell **62:**1143–1151.

33. **Murakami, H., and P. Nurse.** 2000. DNA replication and damage checkpoints and meiotic cell cycle controls in the fission and budding yeasts. Biochem. J. **349:**1–12.

34. **Nurse, P., P. Thuriaux, and K. Nasmyth.** 1976. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **146:**167–178.

35. **Reid, R. J. D., P. Fiorani, M. Sugawara, and M. Bjornsti.** 1999. CDC45 and DPB11 are required for processive DNA replication and resistance to DNA topoisomerase I-mediated DNA damage. Proc. Natl. Acad. Sci. USA **96:**11440–11445.

36. **Rhind, N., and P. Russell.** 1998. The Schizosaccharomyces pombe S-phase checkpoint differentiates between different types of DNA damage. Genetics **149:**1729–1737.

37. **Saka, Y., P. Fantes, T. Sutani, C. McInerny, J. Creanor, and M. Yanagida.** 1994. Fission yeast cut5 links nuclear chromatin and M phase regulator in the replication checkpoint control. EMBO J. **13:**5319–5329.

38. **Saka, Y., and M. Yanagida.** 1993. Fission yeast cut5+, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene rad4+. Cell **74:**383–393.

39. **Shivji, M. K., V. N. Podust, U. Hübscher, and R. D. Wood.** 1995. Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry **34:**5011–5017.

40. **Sugino, A., T. Ohara, J. Sebastian, N. Nakashima, and H. Araki.** 1998. DNA polymerase epsilon encoded by cdc20$^+$ is required for chromosomal DNA replication in the fission yeast Schizosaccharomyces pombe. Genes Cells **3:**99–110.

41. **Verkade, H. M., and M. J. O'Connell.** 1998. Cut5 is a component of the UV-responsive DNA damage checkpoint in fission yeast. Mol. Gen. Genet. **260:**426–433.

42. **Walworth, N., S. Davey, and D. Beach.** 1993. Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. Nature **363:**368–371.

43. **Wang, H., and S. J. Elledge.** 1999. DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **90:**3824–3829.

44. **Wang, Z., X. Wu, and E. C. Freidberg.** 1993. DNA repair synthesis during base excision repair in vitro is catalyzed by DNA polymerase epsilon and is influenced by DNA polymerases alpha and delta in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**1051–1058.

45. **Weinert, T.** 1998. DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. Cell **94:**555–558.

46. **Zou, L., and B. Stillman.** 1998. Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science **280:**593–596.