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Genetics

Schizosaccharomyces pombe cdc20$^+$ encodes DNA polymerase ε and is required for chromosomal replication but not for the S phase checkpoint

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ABSTRACT **In fission yeast both DNA polymerase alpha (pol α) and delta (pol δ) are required for DNA chromosomal replication. Here we demonstrate that *Schizosaccharomyces pombe cdc20*$^+$ encodes the catalytic subunit of DNA polymerase epsilon (pol ε) and that this enzyme is also required for DNA replication. Following a shift to the restrictive temperature, *cdc20* temperature-sensitive mutant cells block at the onset of DNA replication, suggesting that *cdc20*$^+$ is required early in S phase very near to the initiation step. In the budding yeast *Saccharomyces cerevisiae*, it has been reported that in addition to its proposed role in chromosomal replication, DNA pol ε (encoded by POL2) also functions directly as an S phase checkpoint sensor [Navas, T. A., Zhou, Z. & Elledge, S. J. (1995) *Cell* 80, 29–39]. We have investigated whether *cdc20*$^+$ is required for the checkpoint control operating in fission yeast, and our data indicate that pol ε does not have a role as a checkpoint sensor coordinating S phase with mitosis. In contrast, germinating spores disrupted for the gene encoding pol α rapidly enter mitosis in the absence of DNA synthesis, suggesting that in the absence of pol α, normal coordination between S phase and mitosis is lost. We propose that the checkpoint signal operating in S phase depends on assembly of the replication initiation complex, and that this signal is generated prior to the elongation stage of DNA synthesis.**

Initiation of DNA synthesis during the eukaryotic cell cycle requires assembly of multiprotein complexes at chromosomal replication origins (1). Biochemical and genetical approaches, particularly in the budding yeast, have led to the identification of a number of proteins that act in this process (2–4). A multi-subunit protein complex called ORC (for *o*rigin *r*ecognition *c*omplex) has been identified that binds specifically to ARS elements that define replication origins in budding yeast (5–9). Additional classes of proteins, including the CDC6/Cdc18 protein and the MCM (mini-chromosome-maintainance) family of proteins, associate with ORC and are thought to establish replication competence during G_1 by forming prereplicative complexes (10–12, 44, 45). After formation of these complexes, additional protein factors including DNA polymerases (pols) α, δ, and ε, proliferating cell nuclear antigen (PCNA), RF-C, RP-A, and other enzymes of DNA metabolism become involved to bring about primer synthesis and chain elongation. It has also been demonstrated that as cells enter S phase, a checkpoint control is activated that blocks onset of mitosis until DNA replication is complete (13–15). The molecular basis of how DNA replication is monitored and the checkpoint signal is sent to block mitosis remains unclear. In *Saccharomyces cerevisiae*, there is evidence suggesting that pol ε and the associated protein Dpb 11 may play a central role in the checkpoint in response to replication blocks imposed by the DNA synthesis inhibitor hydroxyurea (16, 17). It is still unknown, however, whether pol ε is also required for a checkpoint that ensures normal coordination between completion of S phase and onset of mitosis.

The fission yeast *Schizosaccharomyces pombe* is a good model system for studying DNA replication and the associated checkpoint controls. A number of genes encoding proteins that are required for chromosomal DNA replication have been identified. These include the ORC-related genes *orp1*$^+$ and *orp2*$^+$, the MCM-related genes *nda1*$^+$/*cdc19*$^+$, *cdc21*$^+$, *nda4*$^+$, and *mis5*$^+$, and several further genes, *cdc18*$^+$, *cut5*$^+$, *cdt1*$^+$, *hsk1*$^+$, *pcn1*$^+$, and the DNA polymerases *pol3*$^+$ and *pol1*$^+$ (18). Of particular importance is *cdc18*$^+$, which plays a critical role in controlling initiation of DNA replication, because high levels of Cdc18p drive cells into repeated rounds of DNA synthesis (19). It has also been shown that Cdc18p and Cdc21p interact with the ORC-related protein Orp1p to establish replication competence (20). Analysis of the gene functions that are required for DNA replication suggested that the checkpoint signal blocking mitosis is put in place at an early step in the initiation of DNA replication (21–22) and is not dependent on the expression of genes required later in S phase such as DNA *pol3*$^+$ and *pcn1*$^+$ (23–24). This led us to hypothesize that the checkpoint signal in S phase depends on assembly of the DNA replication initiation complex (22). To identify more gene products acting during DNA replication that may also have a role in establishing the checkpoint signal, we have characterized additional *cdc* mutants in fission yeast that are defective in S phase. One of these is the temperature-sensitive mutant *cdc20-M10*, which blocks early in the cell cycle very near to the initiation of DNA replication (25). Here we report the cloning of *cdc20*$^+$ and demonstrate that it encodes the catalytic subunit of DNA pol ε. Consistent with a role for this enzyme in the replication of chromosomal DNA, we show by fluorescence-activated cell sorter (FACS) analysis that *cdc20* mutants arrest in late G_1 or early S phase after being shifted to the restrictive temperature. Analysis of chromosomes isolated from the *cdc20* mutant by pulsed-field gel electrophoresis suggests that the DNA replication is specifically blocked during the elongation stage of DNA replication. These results support conclusions from genetic work in budding yeast that pol ε is essential for chromosomal replication

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Abbreviations: ORC, origin recognition complex; FACS, fluorescence-activated cell sorter; SV40, simian virus 40; pol α, δ, and ε, DNA polymerase α, δ, and ε.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. Z95397).
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(26–27) but contrast with the conclusions from simian virus 40 (SV40) *in vitro* DNA replication studies, suggesting that DNA polymerase ε is not required for DNA replication (28–29). We have also addressed the potential function of DNA pol ε in the checkpoint control operating in S phase by examining the terminal phenotype of germinating spores deleted for the *cdc20*$^+$ gene. Following germination, spores deleted for *cdc20*$^+$ fail to replicate DNA, but there is no evidence that the S phase checkpoint control operating in these cells is defective. We conclude that, although DNA pol ε is required for an early step in the replication of DNA, it is dispensable for normal checkpoint function during S phase.

MATERIALS AND METHODS

***S. pombe* Methods.** All strains were derived from *972h*$^-$, *975h*$^+$, and *968h*^{90} using standard genetical procedures (30). All media and growth conditions, unless otherwise noted, were as described (31). Cells were prepared for FACS and stained with propidium iodide as previously described (32). The gene corresponding to *cdc20*$^+$ was mapped to chromosome II, between the auxotrophic markers *his4* and *ade1*, using standard genetic methods. Cosmids spanning this region (33–34) were transformed into the strain *cdc20-M10* together with a plasmid containing a selectable marker. A single cosmid, designated 25h2 (34), was identified that could rescue the *cdc20*^{ts} mutant. Cosmid 25h2 was sequenced using a shotgun approach as described (35). To disrupt the *cdc20*$^+$ gene, a 6.2-kb *Xho*I–*Nru*I fragment, containing nearly all the *cdc20*$^+$ coding sequences, was replaced by a single copy of the *ura4*$^+$ gene. The *Sac*I–*Kpn*I fragment derived from pKS-Δcdc20, containing the *ura4*$^+$ gene flanked by *cdc20*$^+$ genomic sequences, was transformed into a diploid with the genotype h$^+$/h$^-$ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216. Transformants able to grow on minimal plates lacking uracil were selected and screened for stability of the *ura4*$^+$ marker. DNA was prepared from a stable transformant that gave rise to two viable and two inviable spores following tetrad analysis. The DNA was digested with a series of restriction enzymes and subjected to Southern hybridization using probes derived from the *cdc20*$^+$ gene. The results of this analysis confirmed that one copy of the two *cdc20*$^+$ genes in the diploid had been replaced with the *ura4*$^+$ marker. For spore germination experiments, spores were derived from diploids with the following genotypes: B, h$^+$/h$^-$ ura4-D18/ura4$^+$ leu1-32/leu1-32 ade6-M210/ade6-M216; C, h$^+$/h$^-$ cdc20$^+$/cdc20::ura4$^+$ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade-M216; D, h$^+$/h$^-$ cdc20-P7/cdc20::ura4$^+$ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216; and E, h$^+$/h$^-$ cdc20$^+$/cdc20::ura4$^+$ leu1-32/cdc20-2::leu1$^+$ ura4-D18/ura4-D18. In B, C, and D, spores were germinated in the absence of uracil to select for *ura4*$^+$ spores only. In E, leucine was also omitted from the media to select for spores containing both the *leu1*$^+$-linked *cdc20-2* allele and *ura4*$^+$-marked Δcdc20.

Pulse-Field Electrophoresis of *S. pombe* Chromosomes. Haploid and diploid cells were used as 1C and 2C standards, respectively. Agarose plugs containing approximately 3×10^8 cells per ml (as determined by OD_{595}) were prepared as previously described (21). Pulse-field gel electrophoresis was carried out in 0.6% chromosomal grade agarose gel (Bio-Rad) using a Bio-Rad CHEF-DRII. The gel was run for 72 hr at 50 V in 0.5 × TAE buffer (40 mM Tris acetate, pH 8.0/1 mM EDTA) with a switch time of 30 min.

Site-Specific Mutagenesis of *cdc20*. Mutations in *S. pombe cdc20* were made by site-directed mutagenesis using the Bio-Rad Muta-gene *in vitro* mutagenesis kit. Oligonucleotides were synthesized that contained the mutagenized site directly adjacent to an engineered *Hin*dIII site. These were used to prime single-strand circular DNA derived from plasmid pKS-B2.7 containing a 2.7-kb Bam fragment spanning the 3′ end of *cdc20*. After primer extension, double-stranded plasmid DNA was transformed into *E. coli* and clones containing the novel *Hin*dIII site were selected and sequenced to confirm the presence of the mutation. The mutated DNA sequence was then used to replace the corresponding wild-type DNA sequence in the plasmid pJK148-*cdc20*, which contains a copy of the *cdc20*$^+$ gene in addition to the *leu1*$^+$ marker. The resulting *cdc20* mutants in plasmid pJK148 were introduced into a diploid strain with the genotype h$^+$/h$^-$ cdc20$^+$/cdc20::ura4$^+$ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 by site-directed integration at the *leu1* locus. Details of this method have been described previously (22). Transformants able to grow on minimal plates lacking leucine were selected and screened for stability of the *leu1*$^+$ marker. Stable transformants were sporulated, and the resulting haploid spores were germinated on minimal plates lacking leucine. The *leu1*$^+$ spores derived from diploids containing the *cdc20-1*, *cdc20-2*, or *cdc20-3* alleles failed to form colonies, whereas those derived from diploids containing the *cdc20*$^+$ gene integrated at the *leu1* locus grew normally (data not shown).

RESULTS

We cloned *cdc20*$^+$ by screening for cosmid clones that spanned the genetically mapped locus after initial attempts to isolate the gene by genetic complementation had proved unsuccessful. One of the spanning cosmid clones could complement the temperature-sensitive *cdc20-M10* mutant, and a fragment from this cosmid was capable of rescuing the mutant by gene conversion. Sequencing of this fragment revealed that it encoded the N-terminal half of a protein with significant homology to DNA pol ε. The observation that this fragment was able to restore wild-type *cdc20* function to the *cdc20-M10* mutant by gene conversion demonstrates that the site of the mutation lies within the N-terminal catalytic domain (see below). The entire cosmid was sequenced and contained the complete ORF corresponding to DNA pol ε (Fig. 1). *S. pombe cdc20*$^+$ is predicted to encode a protein of 2,199 aa that shares more than 40% identity with DNA pol ε from human (36) or budding yeast (37). This homology is more than 60% identical in the N-terminal half of the protein, where many of the sequence elements common to eukaryotic DNA polymerases are located. In the C terminus, the homology is lower (approximately 30% overall), but the zinc finger DNA-binding motifs, which are found in both the human DNA pol ε and budding yeast *POL2*, are also conserved in *S. pombe cdc20*$^+$. These zinc finger motifs have been implicated in the S phase checkpoint control in budding yeast (17).

***cdc20*$^+$ Is Required for Cellular DNA Replication.** To clarify the precise role of DNA pol ε in DNA replication we have used flow cytometry to analyze the DNA content of *cdc20-P7* mutant cells (*cdc20-P7* is a temperature-sensitive mutant) following a shift to the restrictive temperature. When grown at the permissive temperature of 25°C, *cdc20-P7* has a 2C DNA content, which is normal for exponentially growing *S. pombe* cells (Fig. 2*A*, 0 hr). Following a shift to the restrictive temperature of 36°C, cells with a 1C DNA content began to accumulate, suggesting that these cells fail to initiate DNA replication (Fig. 2*A*, 2 hr). After 4 hr at the nonpermissive temperature, all cells were arrested in G_1 or S phase (Fig. 2*A*, 4 hr) and there was no further increase in cell number (ref. 24; data not shown). We also performed pulse-field gel electrophoresis of chromosomal DNA prepared from the *cdc20-P7* mutant. We found that chromosomes isolated from *cdc20* 3 hr after shift-up to the restrictive temperature failed to enter the gel, suggesting that events associated with the initiation of DNA replication had occurred (Fig. 2*B*, lanes 3 and 4; ref. 20).

The C-Terminal End of *S. pombe* DNA pol ε Defines an Essential Domain. In budding yeast it has been reported that

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   1  MPLKTARGAS KYQFRKFNGN YNGKSKSNGR TFAKSTEEVG FNDPMKIVYK
  51  KNEIDRMMGF DSYEGGQPRE AWLLNVHPTV IESTKGNSTL SAVDFYFIQD
 101  DGDTFRCTIP YSPYFYIAAR EGKEALVDDY LKKKFVGLIK STTRIFKEDL
 151  QLKNHIVGYQ KLYIKLVFDN LNDLQAVRKS LMSAVKANSS QQDAVDAYTN
 201  LSSENLNGII ENAFEDPLNH VLDIREYDVP YHSRTLIDLN IRVGQWYTVS
 251  YHEGHVQISL LASRIERAEP TIMAFDIETT KLPLKFPDSS FDKIMMISYM
 301  IDGQGFLITN REIISQNIED FHYTPREEFE GPFIIFNEPD EVGLLHRFFK
 351  HIRSAKPSVI VTYNGDFFDW PFVDARAAFH GLNLTEETGF FRDAEDEYKS
 401  SYCSHMDAFR WVKRDSYLPQ GSQGLKAVTV SKLGYNPIEL DPELMTPYAS
 451  EKPQVLAQYS VSDAVATYFL YMKYVHPFIF SLCNIIPLNP DEVLRKGTGT
 501  LCETLLTVEA CTKNIILPNK HVDASQKFFD GHLLASETYV GGHVESLESG
 551  VFRSDLPTNF NMDPKVYEEL ILQLDKALDF SLTVENNVNV DEIENYEEVR
 601  DSILKKLSDL RDRPKRSEKP RIYHLDVASM YPNIMITNRL QPDSVKDESF
 651  CATCDLNVPN KTCDRRMVWA WRGEYYPAKK GEYHMIYSAL QSERFPGPTP
 701  FSPFRSFQEL SPSEQAAMVQ KRIADYSRKV YHRLYDNTVI ERETIICQKE
 751  NSFYIDTVKS FRDRRYDFKG LQKKWVKQLA AIKEKGGLAE IEEAKKMVVL
 801  YDSLQLAHKV ILNSFYGYVM RKGSRWYSIE MAGITCLTGA TIIQMARQIV
 851  ERAGRPLELD TDGIWCILPE SFPENFEFKK KSGGKVFISY PCVMLNHLVH
 901  EKFTNHQYSA LKDPEKLVYE TTSENSIFFE VDGPYRAMIL PASTEEGKNL
 951  KKRYAVFNFD GSLAELKGFE VKRRGELKLI KDFQSQIFKV FLKGDSLEEC
1001  YQEVAYVADT WLEILFTKGS NLTDDELIEL ISENRSMSKA LSEYGSQKST
1051  SITTARRLAD FLGDQMTKDK GLACRFIISA SPKGRPVAER AVPVAIFFAE
1101  ESVKRHFLRL WLKDNGLYDV DIRDIIDWDY YLKRLGSVVQ KLISIPAALQ
1151  RISNPVTRFP LPDWLQKRVA VLNSKYQQKK IDSIFSLAPT NPSTINNTKV
1201  TDIEDLGSVT HKDKRIVARV TKRKLLQQSG NSEAPVSFEV KPVSFMDGYS
1251  NWLKYAKKKW KYQKQVKLRR RHLIGFQSRQ FTNVLQSSAE VMFENLWHIL
1301  QIRETDVPGI LHAWVIIRNR LTSIRFIVNR KFFVCFKDET LPNVEIEGCL
1351  IEKSNAILPH GSTSDKLFLL EIPEKSYLTE KVSISMIFAH PSVSGIYETR
1401  IEPIERLILE MGSRKRFNNS VPGALGKGFE FGFESKMFTD PSDNDVSYLD
1451  GVEMNYLYAF HFSISNRFVF SLFMPHLKKV EAIIYDKLPG SDMSFPSISK
1501  IYEELRSKFD NLIKESSIEY PDTLSCNVIF SGNERKAYKL IDEKLLQYFS
1551  TKTKNSLLII ESSLPHILKA NVKQIEELPY IMIPRLESNI QSLSWKQHIA
1601  TKMIQHFLAI GSWLFHRIQL SRFSDIPLCN FESDDIQYSI DVVYSRKLKE
1651  HNIILWWNKG PTPDLGGIEK DSILQIASPK DPLEVNNPGA YSNACVDISL
1701  SNLALCSILN SALINDIEGI GDMAALNDNY MTAINDDLEE KLGIHDNIGL
1751  THSLPVLKAL VKTWWNEAAS GNNLADLIIQ HLARWISSSK SYLYSPLLSS
1801  HVEVIMRKTF LQLLSEIKRL GAHIIHASAN KILIKTSKLI VQNAVTYSNY
1851  LLKSIKTLPL FHFLDLNVTE YWDYLLWMDS VNYGGKMVAA NFSATNEEPQ
1901  TVVSWHIKSH LPPIIQPEFQ SWIVEFIEEV YKQKLEKSNT KVGFVRVKNN
1951  NADEDSEIVG SGILKSKLIH PLKRKVAQVR RCFQELQLDE NTREDLKFPK
2001  LPGSFLNYTD GALELVKSIC AVFELSHDLN LEVRFLKKSL LSLLQIQEFS
2051  TQAVFRYPSR RLSLDQIPCK QCGVHQDFDL CLHEHLWPTR DDMGTLVFSD
2101  GWSCSSCNLV YDRWVFEETL VDNLYHQLTL YQLQDLICSK CKTVKQWSLK
2151  ERCSCSGEWV LQLSPTKFRE MLNVYQSVAD FYEFSILQNS VQSILSVLN
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FIG. 1. Sequence of DNA polymerase ε from fission yeast. The ORF is given in single-letter code. Those regions with highest similarity to other eukaryotic DNA polymerases (38) or that comprise a potential exonuclease domain (39) are shown in bold. In *S. pombe*, these include amino acid residues 855–864 (domain I), 590–654 (domain II), 802–843 (domain III), 961–977 (domain V), 765–781 (domain VI), 335–349 (*Exo*I), 360–374 (*Exo*II), and 493–507 (*Exo*III). Region IV is equivalent to *Exo*I and *Exo*II combined. This region overall (from residues 335–977) is 69% and 63% identical to budding yeast POL2 and human DNA polymerase ε, respectively. The conserved cysteine residues in the C terminus of the protein that potentially bind Zn^{2+} are underlined and shown in bold.

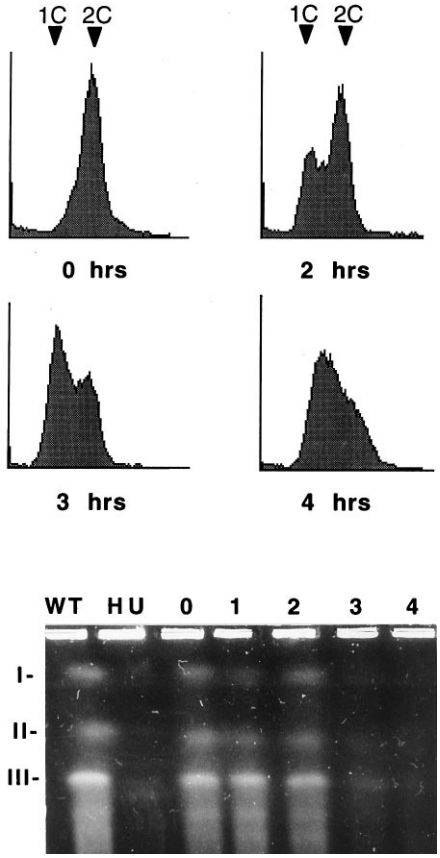

FIG. 2. (*Upper*) DNA content of *cdc20-P7* following a shift to the restrictive temperature. Cells were grown in minimal media at the permissive temperature of 25°C. Cells were then shifted to the nonpermissive temperature of 36°C, and cell samples were collected every hour for 4 hr. DNA content was determined by FACS analysis. The *x*-axis corresponds to DNA content, and the *y*-axis corresponds to relative cell number. The positions of 1C and 2C DNA content are indicated. (*Lower*) Pulse-field gel electrophoresis of *cdc20-P7* chromosomal DNA. Cells collected for FACS analysis in *Upper* were used to prepare agarose plugs for pulse-field gel electrophoresis. I, II, and III indicate the position of *S. pombe* chromosomes 1, 2, and 3, respectively. Chromosomal DNA from hydroxyurea treated wild-type ($972h^-$) cells (lane HU) or wild-type cells ($972h^-$) (lane WT) were used for comparison. Lanes 0–4 correspond to the number of hours cells were grown at the restrictive temperature.

mutations in the gene encoding DNA pol ε (*POL2*) disrupt the checkpoint that inhibits mitosis when DNA replication is blocked by treatment with hydroxyurea (17). These mutations map to the C-terminal tail of DNA pol ε adjacent to the proposed zinc-finger DNA-binding domains. Two mutants were isolated, *pol2-11* and *pol2-12*, that were defective for the checkpoint, and in both cases, a single base-pair change was responsible for a short truncation of the protein (Fig. 3). It was concluded that this domain might serve as a checkpoint sensor in this organism, activating a signal-transduction pathway inhibiting mitosis. To test this hypothesis in fission yeast we constructed similar mutations in *cdc20*$^+$. Site-directed mutagenesis was used to introduce a stop codon at positions 2164, 2169, and 2171 (Fig. 3), which, based on sequence alignment of POL2 and *cdc20*$^+$, are in the same region of the protein as the *S. cerevisiae* mutations *pol2-11* and *pol2-12.* We found that all three mutant alleles were unable to rescue a Δ*cdc20* strain when introduced by site-specific integration at the *leu1* locus, demonstrating that the C-terminal domain of *S. pombe* DNA pol ε is essential for viability. Moreover, analysis of these mutants by spore germination experiments suggests that the C-terminal domain is not essential for S phase checkpoint control (see below).

DNA Pol ε Is Not Essential for the Checkpoint Control Coupling S Phase to Mitosis. To test whether *cdc20*$^+$ was required for the checkpoint control operating in S phase, we disrupted the *cdc20*$^+$ gene by gene replacement with the *ura4*$^+$ marker and examined the phenotype of germinating spores containing the disruption. After germination, cells disrupted for *cdc20*$^+$ arrested with an elongated cell phenotype and showed only a low incidence of aberrant mitoses, <5% (Fig. 4*A*), indicating that the checkpoint control is intact. The same result was also observed when cells were treated with hydroxyurea (Sigma, 11 mM), indicating that the checkpoint control is also functional in the presence of this inhibitor. This result should be compared with the results obtained when cells are disrupted for the gene encoding pol α (encoded by the *pol1*$^+$ gene); in this case, cells enter mitosis in the absence of DNA replication, resulting in a high percentage of aberrant mitoses 10 hr postinoculation (ref. 22; Fig. 4*A*).

The previous experiment was carried out using Δcdc20 spores derived from a *cdc20*$^+$/Δ*cdc20* diploid, so it is possible that some residual Cdc20 protein is carried over into the germinating Δcdc20 spores, which might be sufficient to activate the checkpoint control. During germination, a small percentage (10–20%) of Δ*cdc20* spores replicated their DNA, although this occurred at a much slower rate than the *cdc20*$^+$ control (compare Fig. 4 *B* and *C*). To address this possibility,

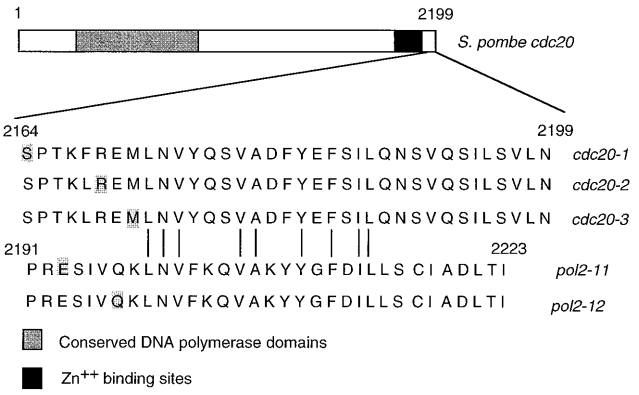

FIG. 3. Site-directed mutagenesis of *cdc20*$^+$. Shaded residues correspond to the positions of the mutations in *S. cerevisiae pol2-11* and *pol2-12*, or to those amino acids modified in *S. pombe cdc20-1*, *cdc20-2*, and *cdc20-3*. In all cases the mutations result in the introduction of a stop codon.

Δ*cdc20* spores were derived from a *cdc20-P7*/Δ*cdc20* diploid and were germinated at the restrictive temperature for the *cdc20-P7* mutant. Any residual protein present in the Δ*cdc20* spores would therefore be temperature-sensitive. These spores germinated and arrested with a 1C DNA content (Fig. 4 *D*), confirming the role of DNA pol ε at the onset of S phase. In addition, none of the cells underwent mitosis, indicating that the checkpoint control was still functional (data not shown). Finally, we germinated spores containing the *cdc20-2* mutation and found that these cells also arrested with a 1C DNA content (Fig. 4*E*) and with an intact checkpoint control (data not shown). Interestingly, whereas a small percentage of Δcdc20 spores derived from the Δ*cdc20*/*cdc20*$^+$ diploid were capable of undergoing a slow partial S phase as noted earlier, in the *cdc20-2* spores derived from the *cdc20-2*/*cdc20*$^+$ diploid, none of the cells replicated their DNA (compare Fig. 4 *B* and *E*). This suggests that the C-terminal truncated form of *cdc20*, which is nonfunctional for DNA replication, acts as a dominant negative allele competing for the residual wild-type protein carried over into some spores, effectively preventing any DNA replication from taking place. Because these cells still arrest with an intact checkpoint, we conclude that the C terminus of DNA pol ε is not required for the checkpoint control operating in S phase.

DISCUSSION

The Role of DNA Pol ε in Chromosomal DNA Replication. We have shown that *S. pombe cdc20*$^+$ encodes the catalytic subunit of DNA pol ε and that this enzyme is required for chromosomal DNA replication. Because *cdc20-M10* (data not shown) and *cdc20-P7* temperature-sensitive mutant cells both arrest with a 1C DNA content when incubated at the restrictive temperature, we conclude that these mutants are defective at an early stage of chromosomal replication. Although the precise role of DNA pol ε at the replication fork is still unclear, this data, combined with the pulse-field gel analysis of chromosomes isolated from the *cdc20-P7* mutant, provide evidence that it plays a critical role in the elongation of nascent DNA chains. That *cdc20* mutants arrest early in S phase is consistent with the possibility that pol ε participates in the switch from primer extension, by DNA pol α/primase, to leading-strand synthesis.

Studies on SV40 DNA replication *in vitro* have indicated that pol ε is not required for DNA replication of plasmid DNA. The mechanism of how DNA is synthesized in the *in vitro* SV40 system may differ from the mechanism operating during cellular-DNA replication. DNA pol ε can be found associated with actively replicating DNA *in vivo* but is not found associated with replicating SV40 chromosomes under similar conditions (40). This suggests that there may be differences

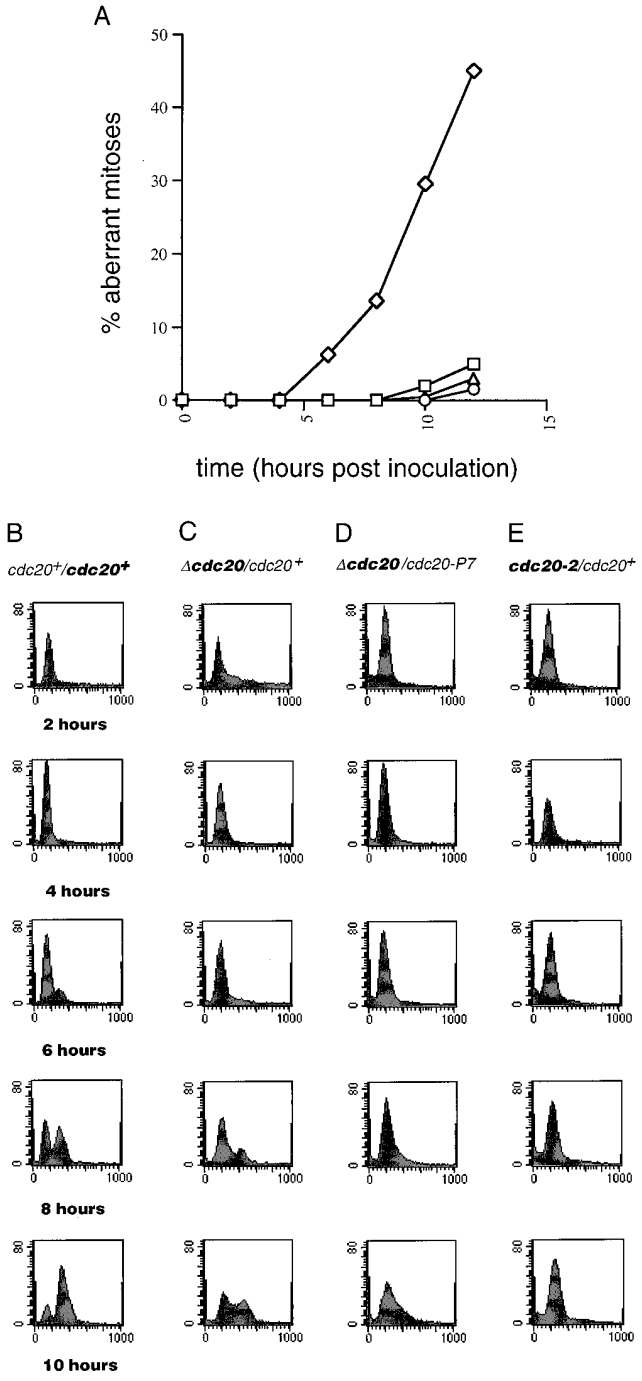

FIG. 4. Germination of spores disrupted for the *cdc20* gene. (*A*) Percentage of aberrant mitoses following germination of wild-type spores (△), spores disrupted for the gene encoding *cdc20*$^+$ (□), *pol1*$^+$ (◇), or *pcn1*$^+$ (○). Aberrant mitoses were scored as the percentage of cells that enter mitosis in the absence of a complete round of DNA synthesis. An aberrant mitosis or "cut" phenotype was confirmed by microscopic analysis, as previously described (21). DNA content of germinating spores containing either the wild-type cdc20$^+$ gene (isolated from a sporolating *cdc20*$^+$/*cdc20*$^+$ diploid strain (*B*), Δ*cdc20* gene (isolated from Δ*cdc20*/*cdc20*$^+$) (*C*), Δ*cdc20* gene (isolated from *cdc20-P7*/*cdc20*$^+$) (*D*), or the *cdc20-2* gene (isolated from *cdc20-2*/*cdc20*$^+$ diploid) (*E*). The positions of 1C and 2C DNA content are indicated. Cells were collected at 2-hr intervals for 10 hr and processed for FACS analysis as described in Fig. 2. Spores containing the wild-type *cdc20*$^+$ gene begin to replicate DNA 5–6 hr after inoculation, and most cells have completed DNA synthesis and are in the G_2 phase of the cell cycle 8–10 hr postinoculation.

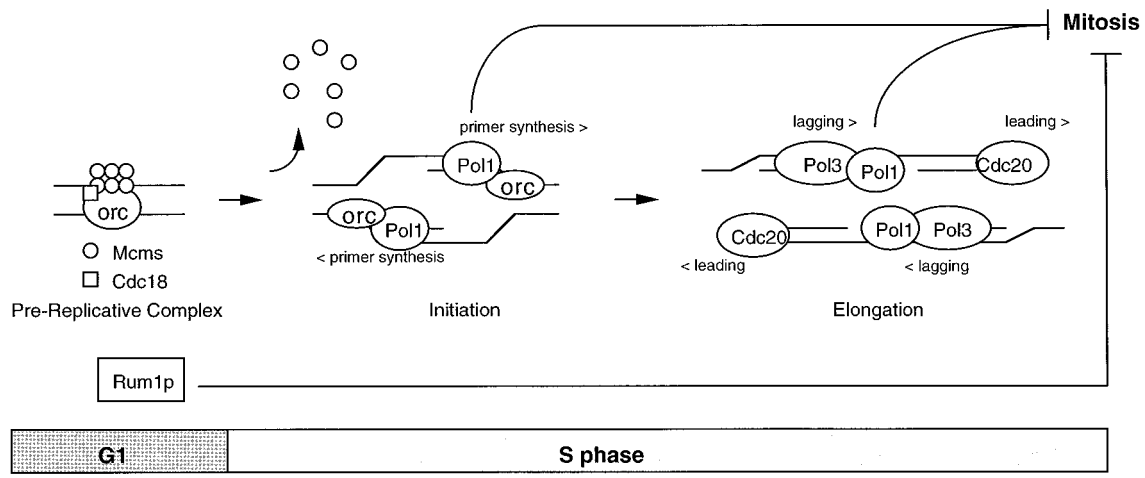

FIG. 5. Assembly of the replication initiation complex is required for a checkpoint control that coordinates completion of DNA replication to the onset of mitosis. In our model, early in G_1 Cdc18p and the Mcm homologs, including Cdc21, Mis5, Nda1/Cdc19, and Nda4, bind to replication origins to form prereplicative complexes. During the G_1 phase of the cell cycle, the presence of the Rum1p prevents cells from prematurely entering mitosis by directly inhibiting the $p34^{cdc2}$ protein kinase (42). At the G_1-to-S phase transition, the Mcm protein complex dissociates and DNA pol α/primase, in addition to other replication factors (not shown), bind to origins of DNA replication to form initiation complexes. Formation of these complexes provides a signal that leads to activation of a checkpoint control, which blocks the onset of mitosis. The binding of both DNA pol ε (encoded by *cdc20*$^+$) and pol δ (encoded by *pol3*$^+$) is then required for the elongation stages of DNA replication. DNA pol ε may function as the leading-strand DNA polymerase, whereas DNA pol δ may function as the lagging-strand polymerase. The continued presence of the DNA polα/primase complex on the lagging strand ensures that the checkpoint control remains active until DNA replication is complete.

between viral and host chromosomal replication, and as a consequence the viral model system may have limitations for understanding eukaryotic DNA replication *in vivo*. Genetic analysis in both *S. cerevisiae* (25–26) and *S. pombe* shows a requirement for DNA pol ε in yeast DNA replication, and the next objective will be to understand how this enzyme interacts with the rest of the replication machinery in S phase.

The Replicative Complex: Establishment of the Checkpoint Sensor. If DNA synthesis is blocked by addition of hydroxyurea or by the use of DNA replication mutants, cells normally arrest in S phase and do not enter mitosis, reflecting the existence of a checkpoint that prevents mitosis in the absence of a complete round of DNA replication (13–15). In *S. pombe*, there is evidence linking the assembly of the replicative complex to the activation of the checkpoint (22). Whether this reflects the existence of a specific protein complex involved in signaling or the appearance of a replication intermediate, such as single-stranded DNA, is still unclear. In the budding yeast *S. cerevisiae*, it has been reported that DNA pol ε (polII) may have a role in sensing DNA replication blocks, specifically through its C-terminal domain (17); these conclusions are based on the observations that mutants defective for polII lose viability and enter mitosis catastrophically following treatment with hydroxyurea. In the case of *S. pombe*, germinated spores deleted for *cdc20*$^+$ are arrested in early S phase and do not enter mitosis, indicating that the checkpoint control is intact in these circumstances. This apparent paradox can be explained if we assume that the checkpoint in response to hydroxyurea, which might involve DNA damage, is different from the normal mechanism that ensures coordination between S phase and M phase. Alternatively, it is possible that *S. cerevisiae* pol ε is not directly involved in sending the checkpoint signal, but that replication complex stability is severely compromised in the *pol2-11* and *pol2-12* mutants after treatment with hydroxyurea. If we assume that the replication complex is essential for checkpoint signaling, as we have proposed for fission yeast (ref. 22; see below), then this would also be expected to lead to a checkpoint failure.

In *S. pombe*, deletion of DNA pol α (encoded by *pol1*$^+$), which is required for initiation, leads to a failure of the S phase checkpoint (ref. 22; Fig. 4*A*). Based on these observations we propose that assembly of the initiation complex, which is distinct from the elongation complex, is required to generate the checkpoint signal (22). We suggest that DNA pol α binds late in G_1, just prior to the initiation of DNA synthesis (Fig. 5), and that this is a necessary step to establish the checkpoint operating in S phase. This checkpoint is not active in G_1, although we expect that prereplicative complexes would have already formed by this time. Instead, cells are prevented from entering mitosis from the G_1 phase of the cell cycle through the action of the $p34^{cdc2}$ inhibitor, Rum1p (41–42). As cells exit G_1, Rum1p is no longer required to restrain mitosis (41), and the S phase checkpoint, which requires the products of the *hus1–5*$^+$, *rad 1*$^+$, *3*$^+$, and *17*$^+$ genes, is activated (43). This may mark the point in the cell cycle when DNA pol α binds to DNA, which may occur precisely at the G_1-to-S phase transition. It will now be important to determine whether binding of DNA polymerases to the prereplicative complex is a key regulatory step in the initiation of DNA synthesis and how, at the molecular level, this step is linked to the activation of the checkpoint control operating in S phase.

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