Rereplication Phenomenon in Fission Yeast Requires MCM Proteins and Other S Phase Genes

Hilary A. Snaith and Susan L. Forsburg

The Salk Institute for Biological Studies, La Jolla, California 92037-1099

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* can be induced to perform multiple rounds of DNA replication without intervening mitoses by manipulating the activity of the cyclin-dependent kinase $p34^{cdc2}$. We have examined the role in this abnormal rereplication of a large panel of genes known to be involved in normal S phase. The genes analyzed can be grouped into four classes: (1) those that have no effect on rereplication, (2) others that delay DNA accumulation, (3) several that allow a gradual increase in DNA content but not in genome equivalents, and finally, (4) mutations that completely block rereplication. The rereplication induced by overexpression of the CDK inhibitor Rum1p or depletion of the Cdc13p cyclin is essentially the same and requires the activity of two minor B-type cyclins, $cig1^+$ and $cig2^+$. In particular, the level, composition, and localization of the MCM protein complex does not alter during rereplication. Thus rereplication in fission yeast mimics the DNA synthesis of normal S phase, and the inability to rereplicate provides an excellent assay for novel S-phase mutants.

THE precise control of DNA replication is of crucial importance for the cell. Every cell cycle, the cell must duplicate its entire genome faithfully, without errors, and exactly once. Failure to replicate a section of DNA will lead to aneuploidy of the daughter cells; rereplication of any sequence will cause increased gene dosage. Therefore there is tight control to ensure the timely and appropriate activation of DNA synthesis.

Work in several systems has shown that the regulated assembly and disassembly of protein complexes at the origin of DNA replication is a key area of S-phase control. The heterohexameric origin recognition complex (ORC) is bound to the origin throughout the cell cycle (Diffley and Cocker 1992; Rowley *et al.* 1995; Liang and Stillman 1997). Prereplicative complexes (pre-RCs) assemble at the ORC-bound origin at the end of mitosis in a reaction that requires the product of the $CDC6/cdc18^+$ gene (Liang *et al.* 1995; Leatherwood *et al.* 1996). The pre-RC then provides the landing pad for the association of a second heterohexameric complex at the origin, the mini-chromosome maintenance (MCM) protein complex (Carpenter *et al.* 1996; Romanowski *et al.* 1996; Donovan *et al.* 1997; Tanaka *et al.* 1997). Thus the origin is primed for DNA replication but requires further activation by Cdc45 and Cdc7 (Owens *et al.* 1997; Zou *et al.* 1997; Bousset and Diffley 1998; Donaldson *et al.* 1998; Zou and Stillman 1998) and the dissociation of Cdc6/Cdc18p (Hua and Newport 1998) before initiating S phase.

Corresponding author: Susan L. Forsburg, Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037-1099. E-mail: forsburg@salk.edu

As cells progress through S phase, the protein complexes assembled during G1 are gradually disassembled. The phosphorylation of certain MCM components (Coué *et al.* 1996; Krude *et al.* 1996) and passage of the replication fork (Aparicio *et al.* 1997; Tanaka *et al.* 1997) apparently remove the MCM protein complex from chromatin. In *Schizosaccharomyces pombe* and metazoans, the MCM proteins remain in the nucleus (S. G. Pasion and S. L. Forsburg, unpublished results; Schulte *et al.* 1995; Krude *et al.* 1996; Okishio *et al.* 1996) but are presumably unable to reassociate with chromatin, due to the absence of Cdc6/Cdc18p (Tanaka *et al.* 1997). The proteolytic degradation of Cdc6/Cdc18p during late S phase and G2 prevents the formation of functional pre-RCs and the refiring of previously replicated origins during a single S phase (Nishitani and Nurse 1995; Cocker *et al.* 1996; Liang and Stillman 1997; Saha *et al.* 1998).

Increasing cyclin-dependent kinase (CDK)/cyclin activity throughout S phase is crucial for ensuring a single round of DNA synthesis per cell cycle. Several experiments have shown the central role of Cdc2 in preventing rereplication in *S. pombe*: (1) An allele of *cdc2*, *cdc2-33*, will perform repeated rounds of DNA synthesis without intervening mitoses (Broek *et al.* 1991), (2) depletion of the mitotic B-type cyclin, Cdc13p, causes massive overreplication (Hayles *et al.* 1994; Fisher and Nurse 1996), and (3) the small CDK inhibitor $rum1^+$ was isolated in a screen to identify genes that resulted in rereplication when overexpressed (Moreno and Nurse 1994). Rum1p selectively inhibits the activity of CDK/Cdc13p (Correa-Bordes and Nurse 1995; Jallepalli and Kelly 1996). Maintaining low CDK activity during S

and G2 phases appears to allow the fission yeast cell to bypass the requirement for passage through mitosis for reinitiation of DNA synthesis.

It has also been found that in certain circumstances, overexpression of the S-phase initiator protein Cdc18p can induce rereplication in *S. pombe* (Kelly *et al.* 1993; Nishitani and Nurse 1995). Cdc18p is normally targeted for ubiquitin-mediated proteolysis in G1/S phase by Cdc2p phosphorylation (Brown *et al.* 1997; Kominami and Toda 1997). However, if Cdc18p is expressed to a high enough level, it apparently titrates the available Cdc2p/cyclin activity and proteolytic machinery, leaving sufficient Cdc18p to reassociate with ORC at the origin and thus trigger another round of DNA synthesis (Nishitani and Nurse 1995). Interestingly, *Saccharomyces cerevisiae* is not sensitive to similar manipulation of Cdc6, which suggests that other pathways may prevent rereplication (Donovan *et al.* 1997; Tanaka *et al.* 1997). However, a newly isolated *cdc6* mutant displays promiscuous initiation of DNA replication and constant association of MCM proteins with chromatin, despite high levels of CDK/cyclin activity, suggesting that this mutant is insensitive to the negative regulation imposed by CDK activity (Liang and Stillman 1997).

Clearly normal cell cycle dependency of S phase is disrupted in rereplicating strains. Is the replication otherwise normal? A crucial assumption is that the S phase that occurs is indeed a typical round of DNA replication that depends upon normal initiation and elongation proteins, rather than being some sort of abnormal DNA synthesis. We have investigated this assumption by examining rereplicating cells for their dependence upon normal S phase genes. Our results suggest that rereplication is a normal S phase that requires all the genes essential for vegetative replication in fission yeast, including the essential MCM proteins.

MATERIALS AND METHODS

General yeast manipulation: All *S. pombe* strains (see Table 1) were maintained on yeast extract plus supplements (YES) agar plates or under selection on Edinburgh minimal media (EMM) with appropriate supplements and using standard techniques (Moreno *et al.* 1991). Mating or diploid sporulation was performed on malt extract (ME) plates for 3 days at 25°. For random spore analysis the samples were treated with 0.5% glusalase overnight at 25° and the spores plated onto suitable selective plates. The Δ*cdc13 p[nmt*.cdc13*$^+$*-leu1*$^+$*]* strains failed to mate on ME. As a result it was necessary to mate this strain on EMM minus nitrogen plus 20% normal supplement concentration. The Δ*cig1* Δ*cig2* double-mutant strain was constructed by tetrad analysis. The *cdc19-HA* strain was constructed as follows: A 2.3-kb *Eco*RI-to-*Sal*I fragment was isolated from plasmid pSLF176 (Forsburg *et al.* 1997), which contains *cdc19-HA*, and subcloned into pJK148 (Keeney and Boeke 1994). The plasmid was linearized with *Bgl*II and integrated into a diploid of FY254/FY261. Stable *leu1*$^+$ haploid isolates were obtained by random spore analysis and backcrossed to *cdc19*ts strains to verify linkage. Production of HA-tagged Cdc19p and absence of wild-type untagged protein was verified by immunoblot. For the spore germination experiments a fresh Δ*cdc13::ura4*$^+$ disruption was constructed. The *cdc13*$^+$ genomic sequence was disrupted by replacing the genomic *Eco*RI fragment within *cdc13*$^+$ with *ura4*$^+$ in pJK148. The *cdc13* disruption cassette was removed from the resulting plasmid as a *Sal*I/*Bam*HI fragment and stably integrated into the *cdc13* genomic locus of a wild-type *ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 his3-D1/his3-D1* diploid strain. We confirmed that the correct gene had been disrupted by Southern analysis.

Induction of Rum1p overexpression: S-phase mutant strains were transformed with either pREP3X-*rum1*$^+$ or the parent vector pREP3X and cultures grown to midlog phase in the presence of thiamine and harvested by centrifugation. Rum1p was induced essentially as described (Moreno and Nurse 1994). A culture was grown to midlog phase in the presence of thiamine. The cells were harvested and the pellets washed four times in an equal volume of thiamine-free EMM and inoculated into fresh EMM to give midlog cultures after 15 hr growth at 25° (permissive temperature). The cultures were then shifted to 36° (restrictive temperature) and growth continued for up to 24 hr. When it was required, cells were treated with 20 mm hydroxyurea during the incubation at restrictive temperature. pREP4X-*rum1*$^+$ was constructed by cloning the *rum1*$^+$ cDNA sequence from pREP3X-*rum1*$^+$ as a 1.5-kb *Xho*I/*Bam*HI fragment into pREP4X (Basi *et al.* 1993).

Depletion of Cdc13p: Plasmid pURnmt(82)*cdc13*$^+$ containing the *cdc13*$^+$ cDNA was the gift of P. Russell. A strain carrying a thiamine repressible copy of the *cdc13*$^+$ gene was constructed by integration of the *cdc13*$^+$ cDNA under the medium strength *nmt* promoter, denoted *nmt** (Basi *et al.* 1993; Forsburg 1993), into the *leu1*$^+$ gene of a Δ*cdc13/cdc13*$^+$ diploid. Briefly, we subcloned the *cdc13*$^+$ cDNA into pREP41 (Basi *et al.* 1993) and then subcloned the *nmt*.cdc13*$^+$ cassette into the pJK148 integrating vector (Keeney and Boeke 1994). The resulting plasmid was linearized with *Nru*I and stably integrated into a Δ*cdc13::ura4*$^+$/*cdc13*$^+$ diploid. The Δ*cdc13/cdc13*$^+$ diploid carrying the stable *nmt*.cdc13*$^+$ integrant was then sporulated to obtain Δ*cdc13::ura4*$^+$ *leu1*$^+$*::nmt*.cdc13*$^+$ haploids. Cdc13p was depleted from cells essentially as described (Fisher and Nurse 1996). Δ*cdc13 nmt*.cdc13*$^+$ cells were grown to midlog phase in thiamine-free EMM, harvested, washed two times in an equal volume nitrogen-free EMM, inoculated into fresh nitrogen-free EMM plus 0.75 μg/ml adenine, and starved for 14 hr at 25°. Following starvation, an equal volume of EMM including 5 mg/ml nitrogen, 5 μg/ml thiamine, and other necessary supplements was added to each culture. The cultures were shifted to the desired temperature and growth continued for up to 24 hr.

Spore germination: Heterologous diploids were constructed using standard genetic techniques. Haploid spores were prepared essentially as described by Forsburg and Nurse (1994). A single diploid colony of each genotype was inoculated into 10 ml YES and grown to midlog phase. The culture was diluted into 200 ml ME to give a starting OD of ~0.05 and allowed to sporulate for 4 days at 25°. The material was harvested, resuspended in 20 ml of 2% glusalase, and incubated for 20 hr at 25°. The resulting spores were extensively washed in yeast nitrogen base (YNB) minus NH_4SO_4 and spun through a 25% glycerol cushion to remove cell debris. Spores were stored at 4° in YNB-N until required. Spores were germinated in EMM plus required nutrient supplements at a starting concentration of 10^7 spores/ml. Samples (1 ml) were removed for flow cytometry every hour.

Flow cytometry: Samples (1 ml) of liquid culture were harvested by centrifugation and fixed in 1 ml of ice-cold 70% ethanol while vortexing (Sazer and Sherwood 1990). Approximately 2×10^6 cells were prepared for FACS analysis.

TABLE 1

Strain list

Strain	Genotype	Reference
FY79	*h*$^+$ Δ*cig1::ura4*$^+$ *ura4-294 leu1-32 ade6-M216*	Bueno *et al.* (1991)
FY194	*h*$^-$ *cdc18-K46 leu1-32 ura4-D18*	Forsburg *et al.* (1997)
FY254	*h*$^-$ *ura4-D18 leu1-32 ade6-M210 can1-1*	Forsburg and Nurse (1994)
FY261	*h*$^+$ *ura4-D18 leu1-32 ade6-M216 can1-1*	Forsburg and Nurse (1994)
FY277	*h*$^-$ Δ*cig2::ura4*$^+$ *ura4-D18 leu1-32 ade6-M210*	Bueno and Russell (1993)
FY310	*h*$^+$ *cdc1-7 ura4-D18 leu1-32 ade6-M216*	Forsburg *et al.* (1997)
FY322	*h*$^-$ *cdc17-K42 ura4-D18 leu1-32 ade6-M216 can1-1*	Forsburg *et al.* (1997)
FY331	*h*$^+$ *cdc20-M10 ura4-D18 leu1-32 ade6-M210*	This study
FY368	*h*$^-$ *cdc23-M36 ura4-D18 leu1-32*	Forsburg *et al.* (1997)
FY421	*h*$^-$ *rad27::ura4*$^+$ *ura4-D18 leu1-32 ade6-704*	This study
FY440	*h*$^+$ *rad4-116 ura4-D18 leu1-32*	This study
FY459	*h*$^+$ *cdc19-P1 ura4-D18 leu1-32 ade6-M216 can1-1*	This study
FY563	*h*$^+$ *cdc10-V50 ura4-D18 leu1-32 ade6-M210*	Forsburg *et al.* (1997)
FY583	*h*$^+$ *cdc22-M45 ura4-D18 leu1-32 ade6-M216*	Forsburg *et al.* (1997)
FY584	*h*$^+$ *cdc25-22 ura4-D18 leu1-32 ade6-M216 can1-1*	This study
FY614	*h*$^+$ *pol*δ*-1(ts) ura4-D18 leu1-32 ade6-M216*	Forsburg *et al.* (1997)
FY628	*h*$^+$/*h*$^-$ Δ*cdc13::ura4*$^+$/*cdc13*$^+$ *ura4-D18/ura4-D18 leu1-32/ leu1-32 ade6-M210/ade6-M216*	Hagan *et al.* (1988)
FY629	*h*$^-$ *cdc24-g1 ura4-D18 leu1-32 ade6-M210*	Gould *et al.* (1998)
FY659	*h*$^+$ *rad3::ura4*$^+$ *ura4-D18 leu1-32 ade6-704*	This study
FY687	*h*$^-$ *top1-170 leu1-32*	NCYC2249
FY688	*h*$^-$ *top2-191 leu1-32*	NCYC2250
FY747	*h*$^+$ *orp1-4 ura4-D18 leu1-32 ade6-M210 can1-1*	This study
FY769	*h*$^-$ *pol1-1 leu1-32*	D'Urso *et al.* (1995)
FY784	*h*$^+$ *cdc21-M68 ura4-D18 leu1-32 ade6-M210*	This study
FY799	*h*$^+$ Δ*cdc19::cdc19-HA::leu1*$^+$ *ura4-D18 leu1-32 ade6-M210*	This study
FY867	*h*$^+$ Δ*cig1::ura4*$^+$ Δ*cig2::ura4*$^+$ *ura4-D18 leu1-32 ade6-M216*	This study
FY868	*h*$^-$ *cdc10-V50* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] ura4-D18 ade6-M210*	This study
FY869	*h*$^-$ *cdc18-K46* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] ura4-D18 leu1-32*	This study
FY870	*h*$^-$ *cdc19-P1* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] ura4-D18 ade6-M210*	This study
FY871	*h*$^-$ *cdc21-M68* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] ura4-D18 ade6-M216*	This study
FY873	*h*$^-$ *pol*δ*-1* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*]ura4-D18 ade6-M216*	This study
FY874	*h*$^+$ *orp1-4* Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] ura4-D18 ade6-M210*	This study
FY875	*h*$^-$ Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*]ura4-D18 ade6-M216*	This study
FY934	*h*$^-$ Δ*cdc19::his3*$^+$*/p[cdc19*$^+$*-LEU2] ura4-D18 leu1-32 ade6-M210 his3-D1*	This study
FY963	*h*$^+$/*h*$^-$ Δ*cdc13::ura4*$^+$/*cdc13*$^+$ *ura4-D18/ura4-D18 leu1-32/ leu1-32 his3-D1/his3-D1 ade6-M210/ade6-M216*	This study
FY993	*h*$^-$ Δ*cdc21::his3*$^+$*/p[cdc21*$^+$*-LEU2] ura4-D18 leu1-32 ade6-M210 his3-D1*	This study
FY1008	*h*$^+$ Δ*cdc13::ura4*$^+$*/p[cdc13*$^+$*-LEU2] ura4-D18 leu1-32 ade6-M216 his3-D1*	This study
FY1033	*h*$^+$ Δ*cdc18::p[nmt*.cdc18*$^+$*-LEU2] ura4-D18 leu1-32 ade6-M216*	This study
FY1049	*h*$^+$ Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$*-leu1*$^+$*] Δcdc18::p[nmt*.cdc18*$^+$*-LEU2] ura4-D18 ade6-M216*	This study
FY1051	*h*$^+$/*h*$^-$ Δ*cdc13::ura4*$^+$/+ Δ*cdc19::his3*$^+$/+ *ura4-D18/ura4-D18 leu1-32/leu1-32 his3-D1/his3-D1 ade6-M210/ade6-M216*	This study
FY1052	*h*$^+$/*h*$^-$ Δ*cdc13::ura4*$^+$/+ Δ*cdc21::his3*$^+$/+ *ura4-D18/ura4-D18 leu1-32/leu1-32 his3-D1/his3-D1 ade6-M210/ade6-M216*	This study

NCYC, National Collection Yeast Cultures.

Cells were rehydrated in 3 ml 50 mm Na Citrate, harvested, treated with 0.5 ml 100 μg/ml RNase A in Na citrate for 2 hr at 37°, and stained in 1 μm Sytox Green overnight at 4° [Molecular Probes, Eugene, OR (Roth *et al.* 1997)]. Analysis was performed on a Becton Dickinson (Franklin Lakes, NJ) FACS Scanner and data was analyzed using Cell Quest software for Macintosh.

Immunofluorescence and DAPI staining: Immunofluorescence procedure was adapted from the previously published protocol (Demeter *et al.* 1995). Briefly, a 50-ml culture was harvested by filtration onto GF-C Whatman paper and the cells fixed in 10% methanol, 3.7% formaldehyde, 0.1 m potassium phosphate, pH 6.5, for 30 min at room temperature. The cells were washed three times in PEM (100 mm PIPES, 1 mm EDTA, 1 mm $MgSO_4$, pH 6.9) and treated with 0.2 mg/ml Novozym 234 (CN Corp), 0.5 mg/ml zymolyase (Seikagaku, Rockville, MD) in PEMS (PEM plus 1.2 m sorbitol) for 5 min at room temperature. Cells were washed three times in PEMS and incubated for 30 min in PEMBAL (1% BSA, 100 mm lysine hydrochloride, 0.1% NaN_3 in PEM). Cells were incubated in primary antibody [anti-HA monoclonal antibody 16B12, BABCO (Berkeley Antibody)] for 14 hr at room temperature and then washed three times for 10 min in 1 ml PEMBAL. Cells were incubated in secondary antibody for 2 hr at room temperature and washed three times for 10 min in PEMBAL. Cells were stained in 1 μg/ml DAPI for 10 min and then washed into water before being heat fixed onto microscope slide. Cells were examined using a Leitz microscope and photographed. Ethanol-fixed cells were rehydrated in water before DAPI staining and examined as described above. Film negatives were digitized using a Nikon Coolscan II by Adobe Photoshop software for Macintosh.

Immunoblotting and immunoprecipitation: Cultures were grown to midlog under the desired conditions and cells harvested by centrifugation. Protein lysates and immunoprecipitation were prepared essentially as described (Sherman *et al.* 1998). Cell lysis buffer contained 50 mm HEPES, pH 7.0, 50 mm potassium acetate, 5 mm magnesium acetate, 100 mm sorbitol, 1 mm ATP, 1 mm DTT, and protease inhibitors. Protein concentration was determined by BCA assay (Pierce, Rockford, IL). Proteins were analyzed by 6% SDS polyacrylamide gel electrophoresis (Protogel, National Diagnostics, Atlanta, GA), followed by transfer to Imobilon P membrane (Millipore, Bedford, MA) and immunoblotting with anti-MCM protein antibodies (Forsburg *et al.* 1997; Sherman *et al.* 1998). The membrane was incubated with 1/2000 dilution of purified antibody in TBS/0.1% Tween 20/5% milk powder for 2 hr at room temperature and with a 1/2000 dilution of goat anti-rabbit HRP-conjugated secondary antibody for 90 min at room temperature, before development of the blot by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). The blots were electronically scanned using a Hewlett Packard (Palo Alto, CA) ScanJet IIcx scanner and analyzed using Adobe Photoshop software for Macintosh.

Pulsed field gel electrophoresis: The method is essentially as described (Kelly *et al.* 1993). Approximately 50 ml cultures were grown in EMM under the desired conditions and harvested by centrifugation. Total cell number was determined by counting using a hemocytometer. Cell pellets were washed in 2 × 10 ml CSE buffer (1.2 m Sorbitol, 40 mm EDTA, 20 mm citric acid, 20 mm Na_2HPO_4, pH 5.6) and incubated in 10 ml 1.5 mg/ml zymolyase (Seikagaku) in CSE for 1 hr at 37°. Cells were harvested by centrifugation and resuspended in 0.5% InCert agarose (FMC) in TSE buffer (0.9 m sorbitol, 10 mm Tris-HCl, 45 mm EDTA, pH 7.5) to give a cell concentration of 3×10^8 cells/ml, and 80-μl plugs formed. Once set the agarose plugs were transferred to 5 ml of ETS buffer (50 mm Tris-HCl, 0.25 mm EDTA, 1% SDS, pH 7.5) for 90 min at 55°. The ETS was replaced with SEP buffer (1 mg/ml proteinase K, 1% lauryl sarcosine, 0.5 m EDTA, pH 9.5) and the incubation continued for 48 hr at 55° with one change of SEP after 24 hr. Plugs were stored at 4° in 1% lauryl sarcosine, 0.5 m EDTA, pH 9.5, until required. Prior to electrophoresis the plugs were washed for 3 × 10 min in TE. Electrophoresis was performed in a contour clamped homogeneous field electric field system tank (CBS Scientific, Del Mar, CA) with 0.6% agarose gel, 0.5 × TAE at 50 V for 72 hr with bidirectional pulse cycles of 1800 sec. The gel was stained with 0.5 μg/ml ethidium bromide and examined on Eagle Eye transilluminator system (Bio-Rad, Richmond, CA).

RESULTS

Rereplication requires S-phase genes

Analysis of Rum1p-induced rereplication: Fission yeast cells undergoing rereplication accumulate DNA in genome equivalents, which suggests that this DNA synthesis represents vegetative replication. However, detailed analysis of the role of known replication factors has not been carried out. We have characterized the dependence of rereplication on S-phase genes with particular attention to normal initiating proteins, including MCM proteins.

Overproduction of the cyclin-dependent kinase inhibitor Rum1p was the first example of dramatic rereplication (Moreno and Nurse 1994). We examined the effect of overproducing Rum1p in cells carrying temperature-sensitive mutations in a variety of S-phase genes to determine whether these genes are required for Rum1p-induced rereplication. As negative controls, we also examined a number of mutants not involved directly in S-phase progression. Each strain was transformed with pREP3X-*rum1*$^+$ (*i.e.*, a plasmid containing the *rum1*$^+$ cDNA under the full-strength inducible *nmt* promoter) or with pREP3X parent vector alone. Rum1p overexpression was induced as described in materials and methods and samples retained for flow cytometry and microscopy. The cells were incubated at the permissive temperature for 15 hr to fully induce the *nmt* promoter prior to the shift to the restrictive temperature (Moreno and Nurse 1994). The cultures were incubated at the restrictive temperature for 6 hr or, as a time course, to inactivate the gene of interest. The effect on rereplication was analyzed by flow cytometry. In all cases where rereplication occurred, it could be blocked by the addition of 20 mm hydroxyurea (data not shown).

From analysis of our results, the mutants can be divided into four major phenotypic classes dependent on their ability to rereplicate in the presence of high levels of Rum1p. Representative FACS profiles are shown in Figure 1 and a summary presented in Table 2. Briefly, a number of mutations had no discernible effect (class A); these were the control strains and contained mutations that do not affect normal S-phase progression, so this was an anticipated result.

Among the known G1/S mutants, we had several phe-

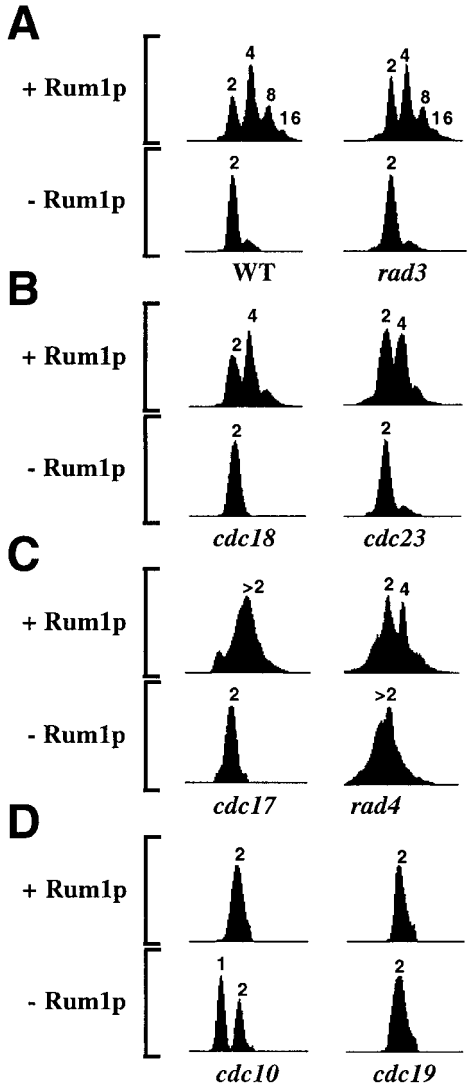

Figure 1.—Representative flow cytometry of the different classes of S-phase mutants in their response to overexpression of Rum1 protein. Rum1p overexpression was performed as described in materials and methods. Flow cytometry analyses are plotted with cell number along the *y*-axis and DNA content on a log scale along the *x*-axis. The numbers above each peak indicate the DNA content relative to known controls. (A) Similar to wild type, (B) slow rereplication, (C) incomplete rereplication, (D) no rereplication.

notypic classes. Some allowed one extra round of replication but much more slowly than in wild type (class B), others prevented full replication but showed a general increase in overall DNA content (class C), and several absolutely blocked rereplication (class D). These classes may reflect the nature of the mutant allele rather than any prediction about mechanism, but all these phenotypes indicate a role for the cognate genes in the rereplication process.

Rereplication induced by depleting Cdc13p requires the same genes as overexpression of Rum1p: The major target of the Rum1p is the cdc2/cdc13^{cyclinB} complex, although if sufficiently overexpressed, Rum1p may also affect other cdk/cyclin complexes present during G1 and S phases, such as Cdc2p/Cig1p and Cdc2p/Cig2p (Correa-Bordes and Nurse 1995; Jallepalli and Kelly 1996). Depletion of Cdc13p also causes massive rereplication (Hayles *et al.* 1994; Fisher and Nurse 1996). We therefore wanted to compare the nature of rereplication occurring when Cdc13p is depleted to that resulting from overexpression of Rum1p. We constructed a Δ*cdc13* strain where the deletion was complemented by a thiamine-repressible form (using the weaker *nmt** promoter) of *cdc13*$^+$ inserted at the *leu1*$^+$ locus. This strain was then crossed with strains with temperature-sensitive mutations in *cdc10*, *cdc18*, *cdc19*, *cdc21*, *orp1*, *pol*δ or *cdc25*. Cdc13p was depleted from cells by growth overnight in EMM minus nitrogen. Cells were induced to reenter the cell cycle by refeeding with nitrogen and thiamine (to repress *de novo cdc13*$^+$ expression) and shifted to the nonpermissive temperature (Hayles *et al.* 1994; Fisher and Nurse 1996). Following this treatment the parent Δ*cdc13* cells developed a hugely elongated and swollen morphology with up to 64C DNA content (Figure 2A, iv; Fisher and Nurse 1996). In contrast, depletion of Cdc13p in the different S-phase mutant backgrounds failed to cause any increase in DNA content. The cells elongated, as expected for *cdc* strains, but the nuclei remained small and compact (Figure 2A, v–viii). The samples were also examined by flow cytometry as shown in Figure 2B. *cdc10* Δ*cdc13 nmt*.cdc13*$^+$ double mutants did not perform any significant DNA synthesis at the restrictive temperature and arrested with a 1C DNA content (Figure 2B, ii). *orp1* Δ*cdc13 nmt*.cdc13*$^+$ mutants behaved in the same way (data not shown). *cdc19* Δ*cdc13 nmt*.cdc13*$^+$ and *cdc21* Δ*cdc13 nmt*.cdc13*$^+$ mutants were able to carry out a single round of DNA replication following release from starvation but were unable to replicate thereafter (Figure 2B, iii and iv). The same was true of *cdc18* and *pol*δ (data not shown). At the permissive temperature, all of the double mutants underwent rereplication in the presence of thiamine and behaved as wild type in the absence of thiamine (data not shown). These results are broadly similar to those obtained from overexpression of Rum1p, confirming that both methods induce rereplication of an apparently normal S phase.

Examining rereplication in null mutants: Cdc18p and MCM proteins are required for both vegetative S phase and rereplication on the basis of results with temperature-sensitive alleles (Figures 1 and 2). However, there is always a concern that *ts* alleles are leaky. For example, the *cdc18-K46* allele has a strikingly different phenotype from the null Δ*cdc18*: The former leads to *cdc* arrest with a 2C DNA content, the latter a checkpoint phenotype with 1C DNA content (Kelly *et al.* 1993). In contrast, the null alleles of *cdc19*$^+$ and *cdc21*$^+$ give phenotypes very similar to the *ts* alleles (Coxon *et al.* 1992; Forsburg and Nurse 1994; Liang *et al.* 1999). To com-

TABLE 2

S-phase mutants characterized by their response to Rum1p over expression

Mutant	Function	Rerep class	Reference
Wild type		A	
cdc25-22	Cdc2p phosphatase	A	Russell and Nurse (1986)
Δ*rad3*[a]	ATM homologue	A	Bentley *et al.* (1996)
Δ*rad27*/Δ*chk1*	Checkpoint kinase	A	Walworth *et al.* (1993)
top1-170	Topoisomerase I	A	Uemura and Yanagida (1984)
top2-191	Topoisomerase II	A	Uemura and Yanagida (1984)
cdc18-K46[a]	Initiation S phase	B	Kelly *et al.* (1993)
cdc23-M36[a]	Function unknown	B	Nasmyth and Nurse (1981)
*pol*δ*-1*	Elongation polymerase	B	Francesconi *et al.* (1993)
orp1-4	ORC subunit	B	Grallert and Nurse (1996)
cdc17-K42[a]	DNA ligase	C	Nasmyth and Nurse (1981)
cdc24-g1	Late S phase?	C	Gould *et al.* (1998)
cdc20-M10	Polymerase ε	C	D'Urso and Nurse (1997)
rad4-116[a]	Initiation S phase	C	Saka and Yanagida (1993)
cdc1-7	Small subunit polδ	D	MacNeill *et al.* (1996)
cdc10-V50[a]	Transcription factor	D	Aves *et al.* (1985)
pol1-1	Polymerase α	D	Damagnez *et al.* (1991)
cdc19-P1[a]	MCM2 homologue	D	Forsburg and Nurse (1994)
cdc21-M68	MCM4 homologue	D	Coxon *et al.* (1992)

The level of DNA synthesis occurring at the restrictive temperature was determined by flow cytometry. [a] Representatives of each class of mutant denoted A–D in the table are presented in Figure 1. A, wild type and mutants that can replicate their DNA to the same extent as wild type; B, mutants with slowed accumulation of DNA in comparison with wild type; C, mutants that are forced to rereplicate by high levels of Rum1p but not in genome equivalents; D, mutants that are unable to rereplicate their DNA.

pare disruption mutants of these essential genes, we used *nmt* shut-off and spore germination procedures.

To study the requirement for Cdc18p we constructed a strain containing null alleles of both *cdc13* and *cdc18*, where both alleles were complemented by integrated wild-type copies of the respective genes under the medium strength *nmt* promoter (see Table 1). Thus in the absence of thiamine this strain behaves like wild type. Conversely, in the presence of thiamine the strain is effectively null for both *cdc13* and *cdc18*. The parent strains Δ*cdc13*, Δ*cdc18*, or Δ*cdc13* Δ*cdc18* were grown in the absence of thiamine (*i.e.*, with expression of Cdc13p and Cdc18p), starved for nitrogen to degrade endogenous Cdc13p and Cdc18p, and then refed with nitrogen in the presence of thiamine to release them into the cell cycle. The recovery from starvation in each strain was monitored by flow cytometry. As shown in Figure 2C, i, the strain deleted for Δ*cdc13* alone produced cells with up to 16C DNA content 8 hr after release from starvation in the presence of thiamine, as expected, while the Δ*cdc18* cells proceeded to cut and display a 1C DNA content (Figure 2C, ii). The double Δ*cdc13* Δ*cdc18* disruption failed to increase its DNA content above 2C, demonstrating that Cdc18p is required for rereplication under these conditions (Figure 2C, iii).

We also examined the response of cells doubly deleted for *cdc13* and the MCM proteins *cdc19*/MCM2 or *cdc21*/MCM4 using a spore germination procedure (data not shown). We have shown previously that the Δ*mcm* alleles have phenotypes similar to those of the temperature-sensitive alleles (Liang *et al.* 1999). In these experiments, a diploid Δ*cdc13::ura4*$^+$ Δ*mcm::his3*$^+$ was sporulated and the spores inoculated into media lacking both uracil and histidine. Spores deleted for only *cdc13* generated up to 16C DNA content 15 hr after inoculation in nutrient media and showed the classic swollen rereplication morphology. In contrast, spores carrying deletions for *cdc13* and either *cdc19*/*mcm2* or *cdc21*/*mcm4* germinated to give a mainly 2C DNA content and a cdc morphology with no swelling. This is consistent with our previous analysis of *mcm* disruption alleles (Liang *et al.* 1999). Together, these results indicate that results with the *ts* alleles are a good indicator of rereplication requirements, even if leaky.

A minimum B-type cyclin activity is required for rereplication: Both overexpression of Rum1p and depletion of Cdc13p induce rereplication by inhibiting or removing the CDK/cyclin during early stages of the cell cycle, consistent with previous reports that Rum1p specifically inhibits CDK/Cdc13p (Correa-Bordes and Nurse 1995). However, there are other B-type cyclins that may specifically act at S phase and be required for Δ*cdc13*-induced rereplication. We wanted to further investigate whether these cyclins were needed for Rum1p-induced rereplication.

Single deletion of either *cig1*$^+$ or *cig2*$^+$ (Bueno *et al.* 1991; Bueno and Russell 1993; Connolly and Beach 1994; Mondesert *et al.* 1996) has no significant effect

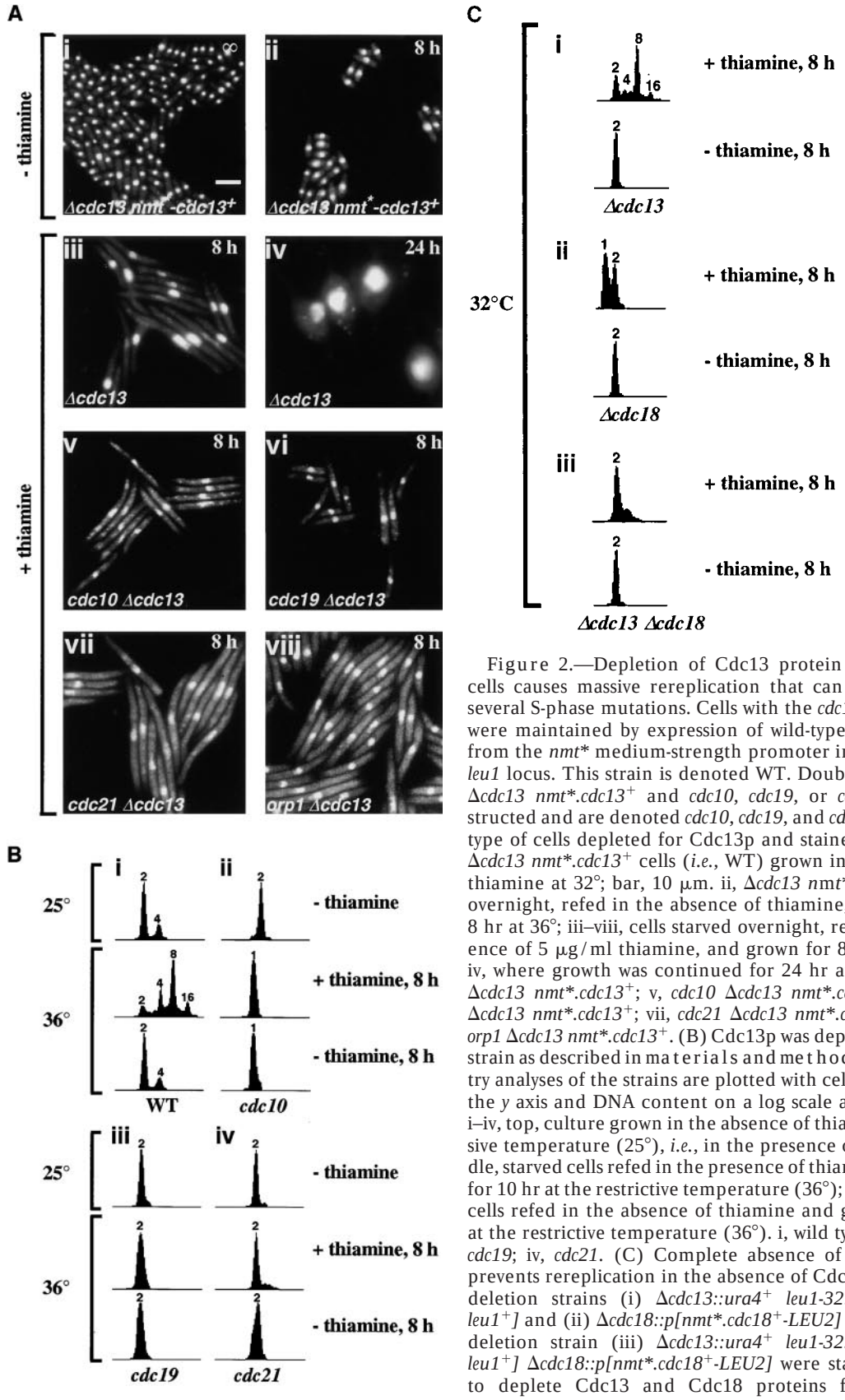

Figure 2.—Depletion of Cdc13 protein from wild-type cells causes massive rereplication that can be blocked by several S-phase mutations. Cells with the *cdc13* gene deleted were maintained by expression of wild-type Cdc13 protein from the *nmt** medium-strength promoter integrated at the *leu1* locus. This strain is denoted WT. Double mutants with Δ*cdc13 nmt*.cdc13*$^+$ and *cdc10*, *cdc19*, or *cdc21* were constructed and are denoted *cdc10*, *cdc19*, and *cdc21*. (A) Phenotype of cells depleted for Cdc13p and stained with DAPI. i, Δ*cdc13 nmt*.cdc13*$^+$ cells (*i.e.*, WT) grown in the absence of thiamine at 32°; bar, 10 μm. ii, Δ*cdc13 nmt*.cdc13*$^+$ starved overnight, refed in the absence of thiamine, and grown for 8 hr at 36°; iii–viii, cells starved overnight, refed in the presence of 5 μg/ml thiamine, and grown for 8 hr, apart from iv, where growth was continued for 24 hr at 36°; iii and iv, Δ*cdc13 nmt*.cdc13*$^+$; v, *cdc10* Δ*cdc13 nmt*.cdc13*$^+$; vi, *cdc19* Δ*cdc13 nmt*.cdc13*$^+$; vii, *cdc21* Δ*cdc13 nmt*.cdc13*$^+$; and viii, *orp1* Δ*cdc13 nmt*.cdc13*$^+$. (B) Cdc13p was depleted from each strain as described in materials and methods. Flow cytometry analyses of the strains are plotted with cell number along the *y* axis and DNA content on a log scale along the *x*-axis; i–iv, top, culture grown in the absence of thiamine at permissive temperature (25°), *i.e.*, in the presence of Cdc13p; middle, starved cells refed in the presence of thiamine and grown for 10 hr at the restrictive temperature (36°); bottom, starved cells refed in the absence of thiamine and grown for 10 hr at the restrictive temperature (36°). i, wild type; ii, *cdc10*; iii, *cdc19*; iv, *cdc21*. (C) Complete absence of Cdc18 protein prevents rereplication in the absence of Cdc13p. The single deletion strains (i) Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$-*leu1*$^+$*]* and (ii) Δ*cdc18::p[nmt*.cdc18*$^+$-*LEU2]* and the double deletion strain (iii) Δ*cdc13::ura4*$^+$ *leu1-32::p[nmt*.cdc13*$^+$-*leu1*$^+$*]* Δ*cdc18::p[nmt*.cdc18*$^+$-*LEU2]* were starved overnight to deplete Cdc13 and Cdc18 proteins from the cells, as described in materials and methods. The duplicate cultures were refed with nitrogen in the presence (top) or absence (bottom) of thiamine and grown for 8 hr at 32°. Flow cytometry analyses of the strains are plotted with cell number along the *y*-axis, and DNA content on a log scale along the *x*-axis.

on the normal vegetative growth of the cells (Bueno and Russell 1993; Connolly and Beach 1994). A strain deleted for both *cig1*$^+$ and *cig2*$^+$ has a slight increase in cells with a 1C DNA content but shows no major delays in growth (Connolly and Beach 1994). We induced overexpression of Rum1p in Δ*cig1*, Δ*cig2*, and the Δ*cig1* Δ*cig2* double-mutant strains from the pREP3X-*rum1*$^+$ plasmid. As shown in Figure 3A, i and ii, both Δ*cig1* and Δ*cig2* strains were able to perform significant levels of rereplication, showing that a single B-type cyclin is able to supply sufficient function to ensure approximately normal regulation. The nuclei of these cells were much larger and stained much more intensely with DAPI than nonreplicating cells, consistent with increased DNA content (Figure 3B, ii and iv). Both strains exhibited a lower accumulation of DNA than is seen in wild type, suggesting that rereplication is slower in the *cig* deletion strains. Strikingly, overexpression of Rum1p in the Δ*cig1* Δ*cig2* double mutant not only failed to induce rereplication but also caused a significant proportion of the cells to arrest with a 1C DNA content (Figure 3A, iii). DAPI staining of these cells confirms much reduced DNA content in comparison with Δ*cig1* and Δ*cig2* cells overexpressing Rum1p (Figure 3B, vi). This is consistent with previous observations showing that *cig* genes are required for rereplication in the absence of *cdc13*$^+$ (Fisher and Nurse 1996). Furthermore, it confirms that rereplication requires some degree of CDK activity and suggests that Cig1p and Cig2p cannot be significantly inhibited by Rum1p, as suggested by biochemical studies (Correa-Bordes and Nurse 1995).

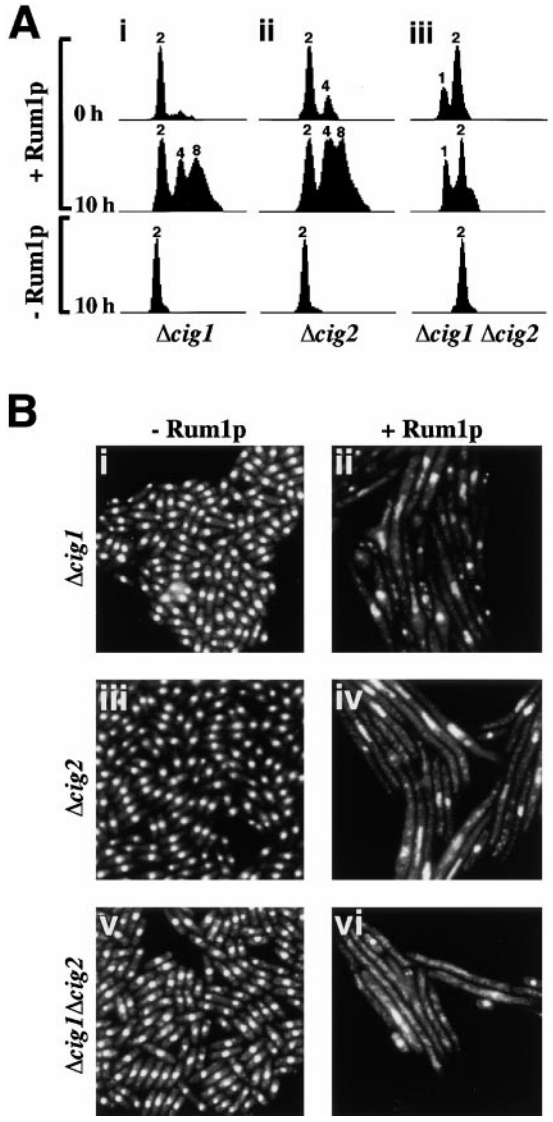

Figure 3.—There is a minimum requirement for B-type cyclins during rereplication. (A) Flow cytometric analysis of Δ*cig1*, Δ*cig2*, and Δ*cig1* Δ*cig2* in the presence and absence of Rum1p overproduction. (i) Δ*cig1*, (ii) Δ*cig2*, and (iii) Δ*cig1* Δ*cig2* strains were transformed with either pREP3X-*rum1*$^+$ (+Rum1p) or pREP3X (−Rum1p). Cultures were grown in the absence of thiamine for 15 hr at 25° and a sample removed for flow cytometry (0 hr). Growth was continued at 36° for 10 hr and the cultures resampled. Flow cytometry analyses are plotted with cell number along the *y*-axis, and DNA content on a log scale along the *x*-axis. Bar, 10 μm. (B) DAPI staining of Δ*cig1*, Δ*cig2*, and Δ*cig1* Δ*cig2* in the presence and absence of Rum1p overproduction. Phenotype of Δ*cig1* (i and ii), Δ*cig2* (iii and iv), and Δ*cig1* Δ*cig2* (v and vi) in the absence (A, C, and E) and presence (B, D, and F) of overexpressed Rum1p. Rum1p was overexpressed in the cells as described in materials and methods. Cells were fixed in 70% ethanol and stained with DAPI.

Analysis of MCM Proteins in Rereplicating Cells

The chromosomes are normal in wild-type rereplicating cells but not in MCM protein mutants: Chromosomes from cells blocked during S phase typically cannot enter a pulsed field gel, presumably due to unresolved replication intermediates (Waseem *et al.* 1992; Kelly *et al.* 1993; Maiorano *et al.* 1996; Liang *et al.* 1999). Thus PFGE can be used as a qualitative measure of chromosome structure. To examine the structure of the chromosomes in cells undergoing rereplication, samples were analyzed by pulsed field gel electrophoresis (PFGE).

Cdc13p was depleted from Δ*cdc13 nmt*.cdc13*$^+$, *cdc19* Δ*cdc13 nmt*.cdc13*$^+$, and *cdc21* Δ*cdc13 nmt*.cdc13*$^+$ cells. The cultures were then refed with nitrogen plus or minus thiamine at the restrictive temperature. The chromosomes were prepared from the wild type and *mcm* mutant cells depleted of Cdc13p, after incubation for 6 hr at 36°. In wild-type cells and Δ*cdc13 nmt*.cdc13*$^+$, the three chromosomes are clearly visible in the gel as shown in Figure 4. This suggests that a significant fraction of chromosomes in wild-type rereplicating cells are not replicating at any given time. If all chromosomes were undergoing active replication, we would not expect to see any chromosomes migrating into the gel. It should be noted, however, that the intensity of the chromosome bands in the rereplicating sample is slightly reduced in comparison to wild type, probably reflecting elevated levels of DNA synthesis in these cells.

In contrast, chromosomes from the *cdc19*/*mcm2* and

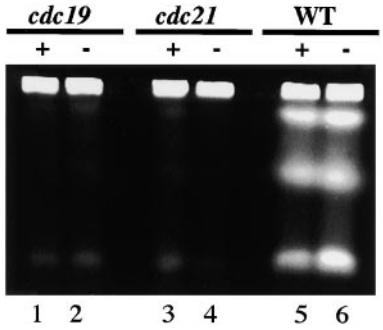

Figure 4.—Pulsed field gel electrophoresis of chromosomes from *cdc19*, *cdc21*, and WT grown in the presence and absence of Cdc13 protein. *cdc19* Δ*cdc13 nmt*.cdc13*$^+$, *cdc21* Δ*cdc13 nmt*.cdc13*$^+$, and Δ*cdc13 nmt*.cdc13*$^+$ strains were starved overnight, refed in the presence and absence of thiamine, and grown at 36° for 6 hr before being harvested for PFGE. Lane 1, *cdc19* plus thiamine (−Cdc13p); lane 2, *cdc19* minus thiamine (+Cdc13p); lane 3, *cdc21* plus thiamine (−Cdc13p); lane 4, *cdc21* minus thiamine (+Cdc13p); lane 5, WT plus thiamine (−Cdc13p); lane 6, WT minus thiamine (+Cdc13p).

cdc21/mcm4 Δ*cdc13 nmt*.cdc13*$^+$ double mutants at the nonpermissive temperature are barely able to enter the gel whether or not Cdc13p is present. This is consistent with previously observed results (Maiorano *et al.* 1996; Liang *et al.* 1999) and suggests that abnormal chromosome structures, presumably from unresolved replication intermediates, occur in rereplicating chromosomes.

The composition of MCM protein complexes does not alter during rereplication: The six fission yeast MCM proteins are present in a heterohexameric complex *in vivo* (Adachi *et al.* 1997; Kubota *et al.* 1997; Sherman *et al.* 1998), and different proteins in the complex bind to other members of the complex with differing affinities (Sherman and Forsburg 1998; Sherman *et al.* 1998). Changes in the composition and localization of the complex may contribute to the control of DNA synthesis. We wanted to determine if there were any differences in levels of individual MCM proteins and if the composition and localization of the complex changed in cells undergoing rereplication compared with wild type. Total protein levels in rereplicating cells increase with DNA (Moreno and Nurse 1994). Therefore, we reasoned that if MCM proteins increase proportionally with DNA, then their levels as a fraction of total protein should be the same. We induced Rum1p overexpression in wild-type cells and prepared total protein lysates from these rereplicating cells and wild-type cells without Rum1p. Equal amounts (as determined by BCA assay) of protein lysate were fractionated by SDS PAGE and immunoblotted for Cdc19p/Mcm2, Cdc21p/Mcm4, Nda4p/Mcm5, and Mis5p/Mcm6 as shown in Figure 5A. There was no increase in the relative levels of MCM proteins in rereplicating cells, suggesting that MCMs increase in proportion to total protein levels, as expected.

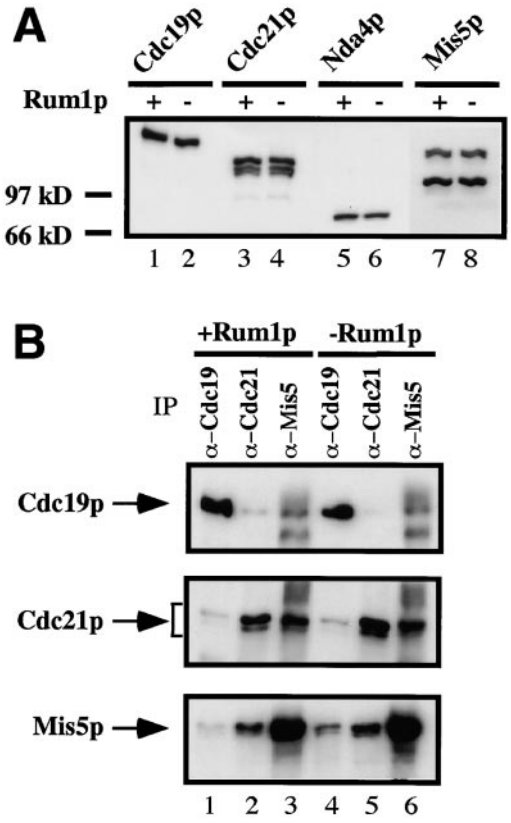

Figure 5.—The relative levels of MCM proteins and MCM complex composition remain unaltered during rereplication. Rereplication was induced by overexpression of Rum1p as described in materials and methods. (A) Total protein lysate was prepared from pREP3X-*rum1*$^+$ and from cells overexpressing Rum1p or carrying empty vector, pREP3X. A total of 10 μg of protein from each lysate was separated by SDS PAGE, transferred to membrane, and immunoblotted for MCM proteins. Lane 1, Cdc19p/Mcm2 levels plus Rum1p; lane 2, Cdc19p/Mcm2 levels minus Rum1p; lane 3, Cdc21p/Mcm4 levels plus Rum1p; lane 4, Cdc21p/Mcm4 levels minus Rum1p; lane 5, Nda4p/Mcm5 levels plus Rum1p; lane 6, Nda4p/Mcm5 levels minus Rum1p; lane 7, Mis5p/Mcm6 levels plus Rum1p; lane 8, Mis5p/Mcm6 levels minus Rum1p. (B) Lysates were prepared from wild-type cells overexpressing Rum1p or carrying empty vector. Immunoprecipitations were carried out with anti-Cdc19p antibodies (lanes 1 and 4), anti-Cdc21p antibodies (lanes 2 and 5), and anti-Mis5p antibodies (lanes 3 and 6) from 300 μg total lysate. Each precipitate was immunoblotted for Cdc19p (top), Cdc21p (middle), and Mis5p (bottom).

We examined the composition of the MCM protein complex by coimmunoprecipitation experiments. Cdc19p/Mcm2, Cdc21p/Mcm4, and Mis5p/Mcm6 were immunoprecipitated from total protein lysates from rereplicating and wild-type cells and immunoblotted for Cdc19p, Cdc21p, and Mis5p (Figure 5B). In wild-type cells Cdc21p and Mis5p are found in a tight complex with Cdc19p more peripherally associated. This is in agreement with previous data (Sherman *et al.* 1998). Comparison between rereplicating and wild-type cells shows that there is no obvious change in the association of complex members during rereplication.

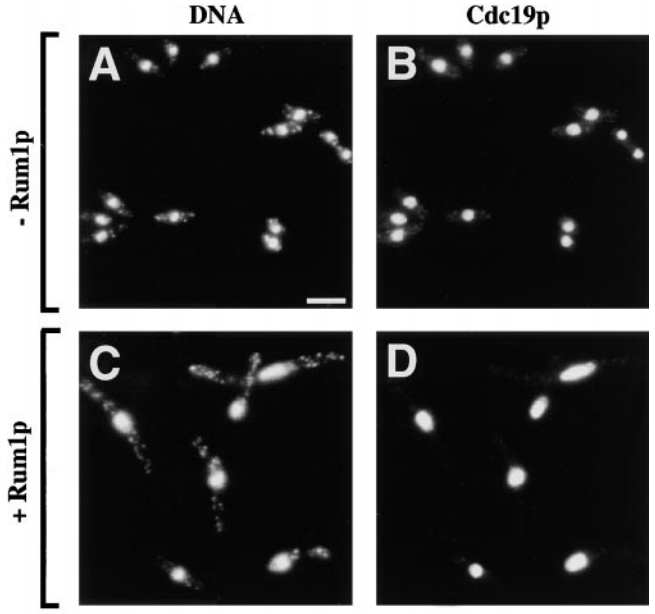

Figure 6.—Cdc19p remains nuclear during replication. Rum1p was overexpressed in strains carrying an HA-tagged copy of *cdc19*$^+$ integrated at the genomic locus as its only source of Cdc19p. (A and C) Samples were prepared for immunofluorescence as described in materials and methods and the DNA stained with DAPI. (B and D) Localization of Cdc19p-HA was determined using 16B12 monoclonal anti-HA antibodies. (A) WT cells without Rum1p overexpression (nonrereplicating). Bar, 10 μm. (B) Same field as in A. (C) WT cells overexpressing Rum1p (rereplicating). (D) Same field as in C.

The MCM proteins remain in the nucleus during rereplication: MCM proteins in *S. pombe*, unlike their counterparts in *S. cerevisiae*, remain in the nucleus throughout the cell cycle and do not relocate to the cytoplasm in wild-type cells (Maiorano *et al.* 1996; Okishio *et al.* 1996; Sherman and Forsburg 1998). We examined the localization of Cdc19p/MCM2 during rereplication as a marker for MCM complex localization in wild-type cells and in several S-phase mutants. The genomic copy of the *cdc19*$^+$ gene was C-terminally tagged with a triple-HA tag and the strains transformed with pREP4X-*rum1*$^+$ or pREP4X. Rum1p overexpression was induced as before and the cells processed for immunofluorescence. The tagged Cdc19p-HA was detected with the 16B12 anti-HA monoclonal antibody. In nonrereplicating wild-type cells, Cdc19p is nuclear (Figure 6, A and B), consistent with previous observations (S. G. Pasion and S. L. Forsburg, unpublished results; Okishio *et al.* 1996). In wild-type cells undergoing rereplication, the Cdc19p remains tightly localized to the nucleus (Figure 6, C and D).

DISCUSSION

This article describes the role of a large panel of genes for rereplication in fission yeast. Wild-type cells can be induced to perform several rapid rounds of DNA replication by overexpressing the CDK inhibitor Rum1p or by depleting the B-type cyclin, Cdc13p. Inhibition of Cdc2p/Cdc13p complexes in these cells is sufficient to prevent entry into mitosis and resets the cell cycle back into pre-START G1 phase. The remaining active B-type cyclins, Cig1p and Cig2p, are required to push the cells back through START and into another round of DNA synthesis (this article; Fisher and Nurse 1996).

Flow cytometry of rereplicating cells shows clear peaks of DNA content, suggesting that DNA synthesis is occurring in discrete genome equivalents, *i.e.*, that the cells fulfill a complete round of DNA replication before reinitiating synthesis. This indicates that rereplication is the same as a conventional S phase, where only a single round of DNA replication can occur per cell cycle. Pulsed field gel analysis of wild-type cells undergoing rereplication shows that their chromosomes have no gross structural abnormalities, further suggesting that the cells are fulfilling a standard S phase.

We analyzed a range of temperature-sensitive mutants and classified them into four phenotypic groups depending upon their response to high levels of the small cyclin kinase inhibitor protein Rum1p. The first category of mutants (A) are those that respond to high levels of the Rum1 protein in the same way as wild-type cells. These were our negative control strains: none of these mutations (*cdc25* and the mitotic topoisomerase mutants *top1* and *top2*) has an effect on normal S phase or on rereplication, although the topoisomerases do affect DNA metabolism. Thus, rereplication is restricted to the G1, S, and early G2 phases of the cell cycle as expected. Further the S-phase checkpoint mutations Δ*rad3* or Δ*chk1* do not affect accumulation of rereplicating DNA; again, this was not surprising because the target of the checkpoint is thought to be the mitotic apparatus, not the replication machinery. This control verifies that no negative feedback on S-phase initiation operates through the checkpoint.

In contrast, all of the mutations known to affect normal S phase also severely affected rereplication. The precise phenotypes of these mutants varied among several phenotypic classes. A few mutants (class B) were able to perform limited rereplication but not to the same extent as the wild-type cells. These mutants are defective in genes that are essential for normal DNA replication. The fact that they are able to carry out some limited rereplication may indicate that the temperature-sensitive allele is leaky. One of this class of mutants is *cdc18*, which is known to be leaky. Cdc18p is phosphorylated by Cdc2p and thereby targeted for ubiquitin-mediated proteolysis (Jallepalli and Kelly 1996; Brown *et al.* 1997; Kominami and Toda 1997; Lopez-Girona *et al.* 1998). Inhibiting activity of the mitotic kinase may stabilize the temperature-sensitive Cdc18p sufficiently to carry out its function in formation of the prereplication complex for one extra round of rereplication. Indeed, in a Δ*cdc18* Δ*cdc13* strain no rereplication is observed.

Other mutants (class C) increase their DNA content upon overexpression of Rum1p but not in genome equivalents. For example, *cdc17* (DNA ligase) and *cdc24* mutants normally arrest at the end of S phase with chromosome fragments activating the DNA damage checkpoint (Nasmyth and Nurse 1981; Johnston *et al.* 1986; Gould *et al.* 1998). Interestingly, most of the mutations of this class act late in S phase, after the bulk of DNA synthesis has occurred. We speculate that these cells, when induced to rereplicate, reenter S phase even though their chromosomes are damaged. Because the DNA synthesis machinery, including the initiating and elongation polymerases, are still active, the mutants accumulate DNA, but because their chromosome integrity is disrupted, it is not in genome equivalents. The DNA replication and DNA damage checkpoints clearly do not affect the ability of these strains to rereplicate.

The final class of mutants (D) are those that fail to perform any rereplication (by flow cytometry) in the presence of high levels of Rum1p. As expected, the Cdc10p transcription factor is required as are two DNA polymerases. The two MCM proteins Cdc19p/Mcm2 and Cdc21p/Mcm4 are absolutely necessary for rereplication, and they arrest with the 2C DNA content typical of *mcm* mutants (Coxon *et al.* 1992; Forsburg and Nurse 1994; Liang *et al.* 1999).

Study of the MCM protein complex in wild-type rereplicating cells revealed that it remains essentially unaltered in comparison with wild-type vegetatively growing cells. Thus, although the cell volume of rereplicating cells is greatly enlarged, there is no alteration in the ratio of MCM proteins to other cellular proteins. Moreover, the association of each member within the complex and the subcellular localization remained the same.

The role of MCMs in rereplication is particularly interesting because some MCMs are proposed to be the targets for CDKs (Hendrickson *et al.* 1996; Krude *et al.* 1996; Maiorano *et al.* 1996). Action of CDK/cyclin complexes apparently serves to displace the MCM complex from the chromatin (Coué *et al.* 1996; Hendrickson *et al.* 1996; Krude *et al.* 1996; Aparicio *et al.* 1997; Tanaka *et al.* 1997). MCM proteins are then prevented from rebinding to chromatin until after cells have completed mitosis. In the absence of CDK activity, we predict that there is nothing to inhibit the MCM proteins from reassociation with the activated origin. This model implies that the activation of the origin by Cdc18p binding is the limiting factor in reinitiating S phase in fission yeast, as in other systems, and may explain why rereplication occurs in genome equivalents with timing that approximates to successive S phases in normally cycling cells.

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