

## Cyclin-Dependent Kinase Inhibits Reinitiation of a Normal S-Phase Program during G<sub>2</sub> in Fission Yeast<sup>∇†</sup>

Lee Kiang,<sup>1\*‡</sup> Christian Heichinger,<sup>1‡§</sup> Stephen Watt,<sup>2¶</sup> Jürg Bähler,<sup>2||</sup> and Paul Nurse<sup>1</sup>

Laboratory of Yeast Genetics and Cell Biology, The Rockefeller University, New York, New York,<sup>1</sup> and  
Cancer Research UK Fission Yeast Functional Genomics Group, Wellcome Trust Sanger Institute,  
Hinxton, Cambridge, United Kingdom<sup>2</sup>

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**To achieve faithful replication of the genome once in each cell cycle, reinitiation of S phase is prevented in G<sub>2</sub> and origins are restricted from refiring within S phase. We have investigated the block to rereplication during G<sub>2</sub> in fission yeast. The DNA synthesis that occurs when G<sub>2</sub>/M cyclin-dependent kinase (CDK) activity is depleted has been assumed to be repeated rounds of S phase without mitosis, but this has not been demonstrated to be the case. We show here that on G<sub>2</sub>/M CDK depletion in G<sub>2</sub>, repeated S phases are induced, which are correlated with normal G<sub>1</sub>/S transcription and attainment of doublings in cell size. Mostly normal mitotic S-phase origins are utilized, although at different efficiencies, and replication is essentially equal across the genome. We conclude that CDK inhibits reinitiation of S phase during G<sub>2</sub>, and if G<sub>2</sub>/M CDK is depleted, replication results from induction of a largely normal S-phase program with only small differences in origin usage and efficiency.**

In every cell cycle, a faithful copy of the genome must be made to ensure its stable inheritance. DNA synthesis is initiated once and only once per cell cycle, and within each S phase, the genome is completely and evenly replicated. Initiation of S phase is contingent upon completion of the previous mitosis, and after S phase, a G<sub>2</sub> cell is inhibited from further initiation (21, 35). DNA synthesis is activated at replication origins along chromosomes; each origin will fire only once or is inactivated by passive replication from a neighboring origin. From the activation of a population of origins, one round of replication is achieved wherein every part of the genome is copied and no portion is copied more than once. Exceptions to the restrictions of one S phase per cell cycle and equal replication across the genome per S phase occur during development in endocycles of megakaryocytes, trophoblasts, and fly nurse and follicle cells (reviewed in reference 12). Within certain endocycles, amplification occurs, in which the genome is replicated unevenly and segments are overreplicated to amplify choriogenes (reviewed in reference 7). In this study we investigated the block to reinitiation of DNA synthesis during G<sub>2</sub> in the fission yeast *Schizosaccharomyces pombe*.

Fission yeast is well suited to the study of this problem

because its cell cycle machinery and the character of its replication origins are conserved with multicellular eukaryotes (22). Replication origins in *Escherichia coli* and the budding yeast *Saccharomyces cerevisiae* have well-defined consensus sequences, and about half of budding yeast origins are used in one out of two cell cycles (15, 34). In contrast, mammalian origins and fission yeast origins lack a well-defined consensus sequence and consist of asymmetric AT-rich stretches of DNA (9, 10, 38). Origin efficiency, that is, the likelihood that an origin will fire in a given S phase, ranges from less than 10% to 76% in fission yeast and is correlated with AT richness (18). Cells that are depleted of the mitotic Cdc13-Cdc2 G<sub>2</sub>/M cyclin-dependent kinase (CDK) complex fail to undergo mitosis and reinitiate DNA synthesis from G<sub>2</sub>, leading to approximate doublings of DNA content as assessed by fluorescence-activated cell sorting (FACS) analysis (14, 17). In budding yeast, fruit flies, and mammals, disrupting G<sub>2</sub>/M CDK activity also leads to rough doublings of DNA content without mitosis (5, 8, 20, 27, 37, 41). This increase in DNA content has generally been assumed to result from a repeated S phase without mitosis. However, for all these organisms, it is not known whether the DNA synthesis that takes place is the consequence of induction of a normal S-phase replication program or represents aberrant unprogrammed DNA synthesis.

We have addressed this question by characterizing the DNA synthesis that occurs in fission yeast when G<sub>2</sub>/M CDK activity is depleted from G<sub>2</sub> cells. We have asked whether DNA synthesis occurs in rounds which are correlated with doublings in cell volume, if there are pulses of G<sub>1</sub>/S gene expression, if replication is equal across the genome, and whether normal mitotic S-phase origins are utilized and conclude that the DNA synthesis which takes place is a largely normal S phase.

### MATERIALS AND METHODS

**Strains and growth conditions.** Standard growth conditions and methods were used (30); all experiments were performed in EMM4S (Edinburgh minimal medium with supplements) unless otherwise stated. 5-Bromo-2-

\* Corresponding author. Mailing address: Laboratory of Yeast Genetics and Cell Biology, The Rockefeller University, 1230 York Avenue, P.O. Box 5, New York, NY 10065. Phone: (212) 327 8475. Fax: (212) 327 8470. E-mail: lkiang@rockefeller.edu.

‡ These authors contributed equally to this work.

§ Present address: Institute of Plant Biology, Zollikerstrasse 107, 8008 Zürich, Switzerland.

¶ Present address: Regulatory Systems Biology Laboratory, Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE, United Kingdom.

|| Present address: Department of Genetics, Evolution and Environment and UCL Cancer Institute, University College London, London WC1E 6BT, United Kingdom.

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deoxyuridine (BrdU) pulse-labeling was performed as described previously (40) using 10-min pulses of 300  $\mu$ M BrdU. SPAP14E8.02 was tagged with green fluorescent protein (GFP) (S65T) at the C terminus using a PCR-based method (4) and is referred to as *tos4-GFP*. *tos4-GFP* strains were grown in EMM4S with 750 mg/liter adenine to reduce autofluorescence (P. Perez, personal communication). *cdc25-22<sup>ts</sup> ade6-M210 tos4-GFP* cells grown to  $1.2 \times 10^6$ /ml were synchronized by a shift to 36.5°C to block cells in G<sub>2</sub>, with cells then released synchronously into the cell cycle at 25°C. To induce rereplication in *cdc13* switch-off (*s/o*) for pulse-labeling,  $\Delta$ *cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 leu1 adh1-hENT his adh1-tk* (from Atanas Kaykov) cells grown at 25°C to  $3.6 \times 10^6$ /ml were filtered, washed four times with Edinburgh minimal medium without NH<sub>4</sub>Cl (EMM-N), and resuspended at  $2.4 \times 10^6$ /ml in EMM-N. After 12 h, 5  $\mu$ g/ml thiamine was added to the culture; 8 h later, NH<sub>4</sub>Cl was added back to the culture with all supplements and the culture was shifted to 32°C to begin the time course. The same protocol was used for  $\Delta$ *cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 tos4-GFP* cells. To impose a *cdc10* block and release in endoreduplicating cells, rereplication was induced as described above in *cdc10-v50  $\Delta$ cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 tos4-GFP* cells at 25°C. Once cells had begun endoreduplication, cells were shifted to 36.5°C at 6 h; they were then released synchronously into endoreduplication to 25°C at 9 h. For a *cdc10* block without rereplication, a culture in the absence of thiamine was grown to  $1.2 \times 10^6$ /ml and shifted to 36.5°C. For origin mapping studies, rereplication was induced in  *$\Delta$ cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 leu1-32ura4-D18* cells by 5 h of nitrogen starvation at 25°C in the presence of thiamine (5  $\mu$ g/ml), followed by a shift to 32°C with or without 11 mM hydroxyurea (HU).

**Microscopy and cell measurements.** Microscopy was performed on a Zeiss Axioplan 2 microscope using a 63 $\times$  objective, Photometrics Coolsnap HQ camera, and Metamorph software, and cell length and width measurements were taken in the ImageJ software program. Cell volume (fl) was calculated by estimating the shape of the cell as a cylinder with a half-sphere at each end. Cells from the 0- to 3-h time points in the *cdc13* *s/o* pulse-labeling experiment were excluded from analysis, since the BrdU signal was not reliably detectable in those cells, potentially due to prior nitrogen starvation. To find the cell volume per nucleus, half of the cell volume for binucleate and septated cells was associated with each nucleus. All cell volumes were binned in discrete intervals, and the percentage of nuclei which incorporated BrdU in each volume cohort was determined. Experiments were performed at least in triplicate unless otherwise noted; representative data are shown.

**Microarray experiments.** Microarray design, DNA preparation, microarray hybridizations, and data acquisition and analysis were as described for HU experiments in reference 19. Data analysis was based on the genome sequence from June 2006. This and current sequence data can be obtained from the Sanger Institute ftp server at [ftp://ftp.sanger.ac.uk/pub/yeast/pombe/Chromosome\\_contigs/](ftp://ftp.sanger.ac.uk/pub/yeast/pombe/Chromosome_contigs/). At least two biological repeats were carried out for each microarray experiment. In some cases, signal ratios for wild-type mitotic S phase have been reassigned in mapping the 904 origins, so efficiencies for 3.8% (34/904) of origins deviate from those in references 19) and 25 (31/904). The *cdc13* experiment without HU was performed twice using open reading frame arrays and once in conjunction with tiling arrays; the reference DNA from G<sub>1</sub>-arrested cells was also used for the self-self control. The reference DNA for all other microarray experiments was from cells blocked in G<sub>2</sub> with 2C DNA content. Data from two repeats were averaged for origin mapping in *cdc13* *s/o*. Percent AT content was defined as the highest percent AT in a 500-bp window (50-bp step) in the intergenic region with at least 65% AT richness that maps to or close to the origin. Intergenic length refers to the number of base pairs in the intergenic region to which the AT-rich island of the origin maps. Efficiency of origins was determined as in reference 19.

**Analysis of prereplicative complex (pre-RC) colocalization with origins.** A custom-made Perl script was used to map signal log<sub>2</sub> ratios from genome-wide chromatin immunoprecipitation-on-chip analysis by Hayashi et al. (16) to each origin in our study. Briefly, among probes with *P* values which conform to the criteria described by Hayashi et al. (>0.025), the script detects the peak signal ratio in the vicinity of the origins by performing iterative searches for a defined threshold within an increasing range from the origin. The data from this analysis using a cutoff signal log<sub>2</sub> ratio of 0.5 and a range of 4 kb about the origin were pooled (from highest to lowest threshold). Our second, more stringent analysis followed a cutoff of signal log<sub>2</sub> ratio greater than 0.8, as in the work of Hayashi et al. Further details are available upon request.

**Microarray data accession number.** Microarray data are available at Array Express under accession no. E-MTAB-105.

## RESULTS

**Single-cell assays for DNA synthesis and G<sub>1</sub>/S gene expression.** A normal mitotic S phase in fission yeast is characterized by the following: (i) a burst of G<sub>1</sub>/S Mlu1 cell cycle box binding factor (MBF)-dependent gene expression, (ii) the initiation of DNA synthesis at a critical cell size, (iii) the use of a specific set of replication origins, and (iv) equal replication across the genome. To investigate whether the DNA synthesis induced when G<sub>2</sub>/M CDK is depleted from G<sub>2</sub> cells exhibits these characteristics, we have used single-cell assays for DNA synthesis and G<sub>1</sub>/S gene expression.

DNA synthesis was monitored in single cells by BrdU pulse-labeling using a strain that expresses the human nucleoside transporter (*hENT*) and herpes simplex thymidine kinase (*tk*). This allows the uptake and conversion of a nucleoside analog label to a nucleotide label and its incorporation into cells undergoing DNA synthesis. Visualization of newly replicated DNA is monitored after fixation by immunofluorescence (40). To validate this method, an asynchronous wild-type culture was pulsed with BrdU for 10 min and signal was detected in septated and recently divided cells which were in S phase (Fig. 1A). Since cell cycle progression is coupled to growth, we measured the cell volume per nucleus of labeled fixed cells, considering binucleate and septated cells as two nuclei within a single cell (see Materials and Methods). The percentage of BrdU-positive cells reached a maximum at about 44 fl (cells shrink due to fixation and processing for immunofluorescence and so are smaller than live cells), and the peak was asymmetrical (Fig. 1A and Table 1). The smallest cell volume per nucleus at which cells were labeled was observed for the cohort of 37 fl, where 50% of the population already was labeled. The 37-fl cohort consisted of binucleate cells which had just completed mitosis. This result is consistent with the fact that there is only a very short G<sub>1</sub> phase in wild-type fission yeast (28) and S phase is initiated soon after completion of mitosis in binucleate cells.

In the normal wild-type mitotic cell cycle, the critical cell size threshold for entry into S phase is cryptic, since newly divided cells are above the size threshold (31), so the critical size is revealed only in a *wee1* mutant which reduces cell size at division. In cells lacking *Wee1*, newly divided cells are small and G<sub>1</sub> is lengthened, since cells must grow to reach the minimum size required for S phase. Therefore, we used temperature-sensitive *wee1-50* cells which are advanced into mitosis to determine if the single-cell assay could detect the reduced cell size for S phase in these cells (31). *Wee1-50* cells growing exponentially at 32°C have a longer G<sub>1</sub> phase than wild-type cells (data not shown and Fig. 1C) and undergo S phase at a reduced cell volume (Fig. 1A), and as expected, the cell volume cohort with maximal labeling was smaller than wild-type cells at about 28 fl (Fig. 1A and Table 1). We conclude that this assay can be used to monitor DNA synthesis and to determine the cell volume at which cells undergo DNA synthesis.

We next developed a single-cell assay to monitor G<sub>1</sub>/S MBF-dependent gene expression in live cells. Factors required for DNA replication expressed at G<sub>1</sub>/S include *Cdc18*, *Cdt1*, *Cig2*, and *Cdc22*, under transcriptional control of MBF, which contains *Cdc10*, *Res1*, *Res2*, and *Rep2* (reviewed in reference 3). Since these MBF-dependent gene products are thought not to

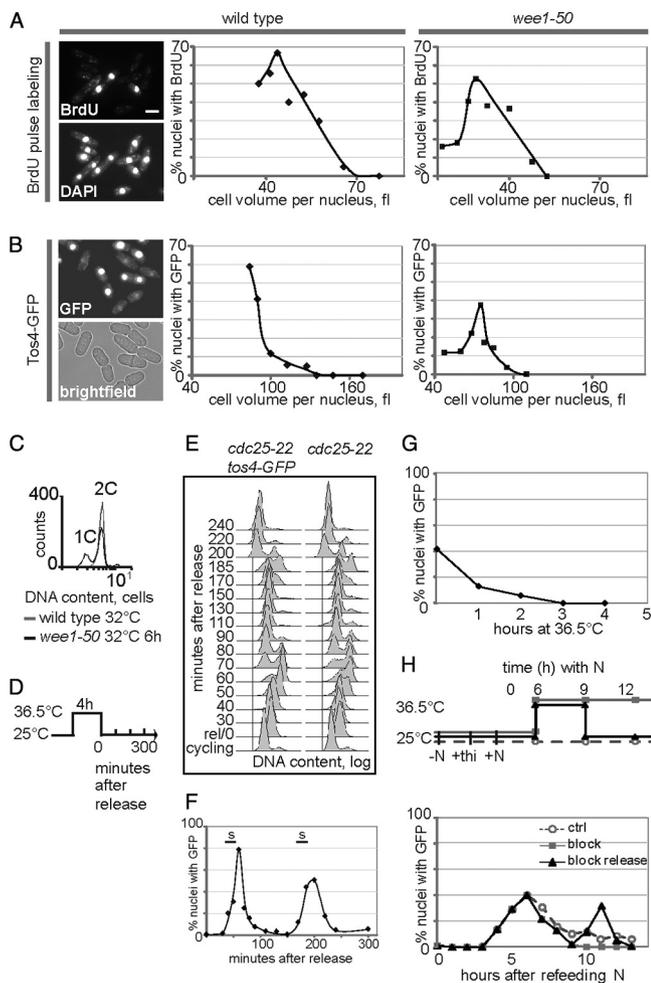


FIG. 1. Single-cell methods for studying DNA synthesis and MBF-dependent gene expression. (A) BrdU pulse-labeling of wild-type and *wee1-50* cells. Left panels: a *leu1-32 his7-366 pJL218 (his7 adh1-*tk*) pFS181 (leu1 adh1-hENT1)* culture at 32°C was pulse-labeled with BrdU. BrdU was detected by immunofluorescence and DNA visualized by 4',6'-diamidino-2-phenylindole (DAPI) staining. The scale bar is 5  $\mu$ m long. The percentage of BrdU-positive nuclei was plotted as a function of fixed cell volume per nucleus. Right panel: a *wee1-50 leu1-32 his7-366 pJL218 (his7 adh1-*tk*) pFS181 (leu1 adh1-hENT1)* culture at 25°C was shifted to 32°C for 6 h. Cells were pulse-labeled with BrdU. (B) MBF-dependent gene expression in wild-type and *wee1-50* cells. The cell volume per nucleus with peak Tos4-GFP signal was determined. Left panels: an *ade6-M210 tos4-GFP::KanMx6* culture at 32°C. The percentage of nuclei with a strong GFP signal was plotted as a function of cell volume per nucleus. Right panel: a *wee1-50 ade6-M210 tos4-GFP::KanMx6* culture at 25°C was shifted to 32°C for 6 h. The percentage of nuclei with a strong Tos4-GFP signal was plotted as a function of cell volume per nucleus. (C) FACS analysis of wild-type cells at 32°C and *wee1-50* cells shifted from 25°C to 32°C for 6 h. *Wee1-50* cells show a 1C peak. (D) Experiment schematic: a *cdc25-22 ade6-M210 tos4-GFP::KanMx6* culture at 25°C was shifted to 36.5°C for 4 h and released at 25°C. (E) FACS analysis: *cdc25-22 tos4-GFP* cycled similarly to *cdc25-22* alone. (F) The percentage of nuclei with strong Tos4-GFP signal was plotted as a function of time, peaking at the end of S phase. Tos4-GFP localizes to the nucleus periodically during the cell cycle. (G) A cycling *cdc10-v50  $\Delta$ cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 tos4-GFP::KanMx6* culture in the absence of thiamine at 25°C was shifted to 36.5°C for 4 h. The percentage of nuclei in the culture with a strong Tos4-GFP signal was assessed every hour. (H) Rereplication was induced in a *cdc10-v50  $\Delta$ cdc13::ura4<sup>+</sup> pREP45 cdc13<sup>+</sup> ade6-704 tos4-GFP::KanMx6* cul-

ture. At 6 h after rerefeeding nitrogen, half of the culture was shifted to 36.5°C for 3 h (gray line, block) while half continued to rereplicate at a permissive temperature (dashed line, control). At 9 h, half of the blocked culture was released to 25°C (black line, block release). The percentage of nuclei with Tos4-GFP was determined at each time point.

give an easily detectable signal in live cells when fluorescently labeled (S. Kearsley, personal communication), we used the nonessential gene SPAP14E8.02, related to *TOS4* of budding yeast, as a marker for G<sub>1</sub>-S gene expression. This gene is periodically expressed in the cell cycle, peaking at G<sub>1</sub> (36). It has MCB1 and MCB2 (Mlu1 cell cycle box) motifs, bound by MBF (36). We constructed a strain in which endogenous SPAP14E8.02 was tagged with a single GFP (S65T) at its C terminus, and we refer to the construct here as *tos4-GFP*. To determine when Tos4-GFP signal peaks in the cell cycle, a culture was synchronized using the *cdc25-22* temperature-sensitive mutation (13) (Fig. 1D). Cells were blocked in G<sub>2</sub> at the restrictive temperature of 36.5°C and then released at 25°C, allowing the population to progress synchronously through the cell cycle. The percentage of cells with GFP signal was monitored over two cell cycles, and DNA content was assessed by FACS analysis (Fig. 1E). Fluorescent protein was seen as a high-intensity signal localized to the nucleus in S phase which decreased in G<sub>2</sub> (Fig. 1F). This was consistent with a peak in message at G<sub>1</sub> (36) and the ~25-min time period needed for GFP (S65T) maturation, which delays the appearance of the protein until later in the cell cycle (19). To verify that *tos4-GFP* is MBF dependent, an exponentially growing culture of *cdc10-v50 tos4-GFP* cells at 25°C was shifted to 36.5°C to inactivate the temperature-sensitive mutant Cdc10 (26, 32). As cells accumulated in early G<sub>1</sub> over 4 h, the Tos4-GFP signal disappeared, indicating that *tos4* expression depends on Cdc10 and thus the MBF complex (Fig. 1G). In contrast, the Tos4-GFP signal was readily observed in control cells with active MBF at 36.5°C (data not shown). A wild-type strain with *tos4-GFP* had a normal cell size, generation time, and DNA content (data not shown). The fluorescent signal was seen in the nuclei of separated cells which are in S phase and recently divided cells which are in S phase and early G<sub>2</sub> (Fig. 1B). The smallest cell volume (per nucleus) was represented by the 90-fl cohort, which was already 60% labeled (Fig. 1B and Table 1). Thus, Tos4-GFP levels oscillate in the normal cell cycle, and the appearance of the protein depends upon MBF. Labeling then gradually declined, in agreement with peak *tos4-GFP* transcription occurring immediately after mitosis. There was a peak in *wee1-50* cells at 75 fl; this smaller size was as expected, given that *wee1-50* cells replicate at a smaller size than wild-type cells (Fig. 1B and Table 1). We conclude that the Tos4-GFP fusion can be used to monitor MBF-dependent gene expression and the cell size at which it occurs.

**DNA synthesis and MBF-dependent gene expression in the *cdc13* switch-off strain.** Cdc13 is the G<sub>2</sub>/M cyclin B which associates with the protein kinase Cdc2 to form the G<sub>2</sub>/M CDK in fission yeast cells. In the *cdc13* s/o strain, the *cdc13* gene is under the control of the thiamine-repressible *nmt41* promoter. Cdc2-Cdc13 CDK is depleted after thiamine addition, leading

ture. At 6 h after rerefeeding nitrogen, half of the culture was shifted to 36.5°C for 3 h (gray line, block) while half continued to rereplicate at a permissive temperature (dashed line, control). At 9 h, half of the blocked culture was released to 25°C (black line, block release). The percentage of nuclei with Tos4-GFP was determined at each time point.

TABLE 1. Volume per nucleus at peak BrdU incorporation or Tos4-GFP signal for successive DNA doublings<sup>a</sup>

Strain description	Vol per nucleus (fl) at peak BrdU incorporation				
	1C→2C	2C→4C	4C→8C	8C→16C	16C→32C
Wild type, BrdU	44				
<i>wee1-50</i> , BrdU	28				
<i>cdc13 s/o</i> , BrdU	NA <sup>b</sup>	50	100	200	400
Wild type, <i>tos4-GFP</i>	90				
<i>wee1-50 tos4-GFP</i>	75				
<i>cdc13 s/o tos4-GFP</i>	95	170	290	600	

<sup>a</sup> Fixed cell volumes are given for the first three strains. BrdU strains contain *tk* and *hENT*. For the bottom three strains, live cell volumes are given.

<sup>b</sup> Not scored. See Materials and Methods for details of *cdc13 s/o* pulse-labeling and calculation of cell volumes.

to DNA synthesis in G<sub>2</sub> cells (14, 17). Previous experiments with the *cdc13 s/o* strain established that continued DNA synthesis in the absence of mitosis occurs most efficiently in cells which have been nitrogen starved, treated with thiamine, and then returned to growth medium in the presence of thiamine so that cells lack Cdc13 when they reach G<sub>2</sub> (J. Hayles, personal communication) (14) (see Materials and Methods). During the subsequent 11 h of growth, the DNA content in the majority of cells increased from 2C to 32C. To determine if DNA synthesis occurred in discrete intervals that correlated with cell volume increase, samples were taken from the *cdc13 s/o tk hENT* culture every hour and pulsed with BrdU. Cells from each time point were examined for BrdU incorporation into the nucleus (Fig. 2A). The percentage of labeled cells remained essentially constant over time (Fig. 2B) (Materials and Methods). When cells from all time points were ordered according to volume, similar to the assay shown in Fig. 1A, three peaks of DNA synthesis and a partial fourth peak were revealed (Fig. 2C). DNA synthesis peaked at fixed cell volumes of 50, 100, and 200 fl, with the partial fourth peak at 400 fl (Fig. 2C and Table 1). These data indicate that the *cdc13 s/o* cells undergo discrete intervals of DNA synthesis in the absence of mitosis and do so periodically approximately with each cell volume doubling. Comparison of the cell size data and FACS profile from each time point showed, respectively, that the peak at 50 fl corresponded to the transition from 2C to 4C, 100 fl to the 4C-to-8C transition, 200 fl to the 8C-to-16C transition, and 400 fl to the 16C-to-32C transition (Fig. 2 and Table 1). These volumes are multiples of about 25 fl, roughly the size at which cells lacking Wee1 activity undergo S phase. We conclude that DNA synthesis is periodic and is correlated to cell size doubling in the *cdc13* switch-off strain. The fact that reinitiation of DNA synthesis occurred roughly at multiples of the normal minimal size for S phase suggests that a dependence of initiation upon cell size increase is maintained.

To determine whether G<sub>1</sub>-to-S-phase MBF-dependent gene expression was also periodic and accompanied the periodic DNA synthesis, we used the *cdc13 s/o* strain containing the *tos4-GFP* marker. We first confirmed that Tos4-GFP was MBF dependent in the absence of Cdc13 by inducing rereplication in a *cdc13 s/o tos4-GFP* strain containing the *cdc10-v50* mutation. A rereplicating culture was shifted to 36.5°C, and the Tos4-GFP signal was observed to disappear as cells blocked in G<sub>1</sub>;

upon release to 25°C, Tos4-GFP reappeared, demonstrating its dependence on MBF in rereplicating cells (Fig. 1H). During the experimental time course, nuclear Tos4-GFP appeared periodically during successive DNA doublings and four periods of nuclear Tos4-GFP signal coincided with the four DNA doublings (Fig. 3 and Table 1). Therefore, there are periodic rounds of both DNA replication and G<sub>1</sub>/S transcription during the time course. These data also indicate that MBF-dependent gene expression does not require passage through mitosis. We conclude that in the *cdc13 s/o* strain, G<sub>1</sub>/S-type MBF-dependent gene expression is periodic and each round of DNA replication is associated with a period of gene expression. These characteristics indicate that a normal S-phase replication program is induced from G<sub>2</sub> when the G<sub>2</sub>/M CDK is removed.

#### DNA replication across the genome and replication origins.

In a normal mitotic S phase, the genome is uniformly replicated with no areas amplified relative to other regions, so there is an equal copy number of each portion of the genome. To determine whether equal rounds of replication or local ampli-

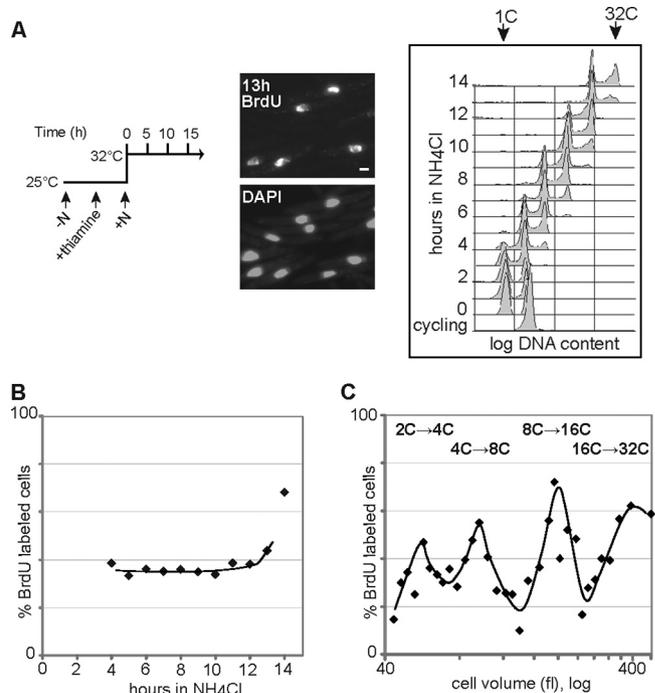


FIG. 2. Periods of DNA synthesis correspond to cell volume doublings in *cdc13* switch-off. (A) BrdU pulse-labeling of cells lacking Cdc13. An exponentially growing  $\Delta cdc13::ura4^+$  *pREP45 cdc13<sup>+</sup> ade6-704 leu1 adh1-hENT his adh1-tk* culture was filtered and resuspended in medium lacking nitrogen. After 8 h, thiamine was added, and after an additional 12 h, nitrogen was replenished and the culture shifted to 32°C. Cells depleted of the Cdc13 cyclin were pulse-labeled with BrdU during a time course as cells underwent DNA doublings from 1C to 32C. DNA was visualized by 4',6'-diamidino-2-phenylindole (DAPI) staining, and BrdU was detected by indirect immunofluorescence. Cells from 13 h after replenishment of nitrogen are shown. The scale bar is 5  $\mu$ m long. (B) The percentage of BrdU-positive cells was plotted as a function of time. (C) Cells from all time points were binned according to fixed cell volume, and the percentage of cells with incorporated BrdU in each bin was plotted as a function of cell volume. Peaks in the percentage of BrdU-positive cells corresponded to DNA doublings.

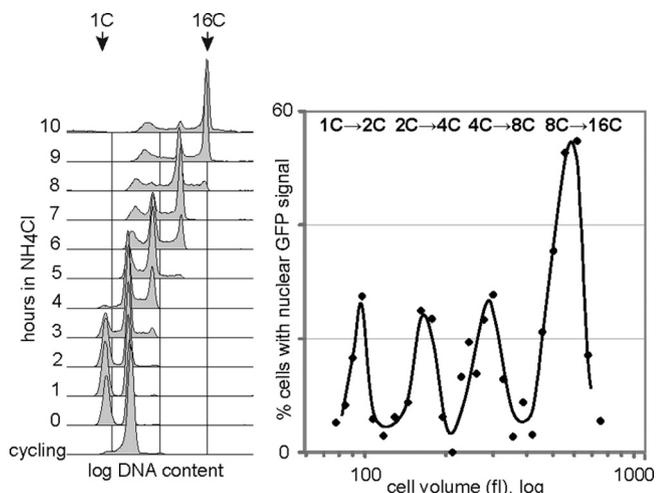


FIG. 3. MBF-dependent gene expression is periodic in *cdc13* switch-off. Nuclear signal of Tos4-GFP in the absence of *cdc13*. Left panel:  $\Delta cdc13::ura4^+$  *pREP45 cdc13^+ ade6-704 tos4-GFP::KanMx6* cells depleted of Cdc13. DNA content increased to 16C. Right panel: the percentage of cells with strong nuclear GFP as a function of cell volume.

fication occurred in the *cdc13* s/o strain, we assayed genomic DNA from cells which had increased their DNA content to 16C to 32C. Using microarrays, we measured the relative DNA content across the genome, using DNA from control G<sub>1</sub>-arrested cells as a reference. This experiment revealed that replication was essentially equal across the genome, with no

region becoming significantly amplified to a higher copy number than any other (Fig. 4). The only possible exceptions are regions very close to the telomeres, where there are slight deviations from the self-self control (Fig. 4). We conclude that although S phase occurs in the absence of mitosis, replication is restricted to discrete and essentially full rounds of genome doubling. This establishes *cdc13* s/o as a system of endoreduplication wherein the genome is duplicated in complete rounds without mitosis as opposed to rereplication or amplification in which aberrant refiring of origins leads to partial reduplication of the genome (reviewed in references 1 and 11).

Since the genome was found to be essentially evenly replicated, as would be expected if each round of replication corresponded to a normal S phase, we investigated whether this was the result of a normal replication program at the level of origin firing. We asked whether S-phase origins are used to reduplicate the genome and whether origins are used with the same efficiency as in wild-type cells. The replication origin profiles from the fission yeast wild-type cell cycle were identified in our previous study (18) using synchronous cell populations treated with HU, which confines DNA synthesis to the vicinity of fired origins (see Materials and Methods). We remapped the 904 mitotic S-phase origins (401 “strong” origins and 503 “putative weaker” origins described by Heichinger et al.), regarding origins within clusters as distinct origins, and assigned each origin the highest signal ratio value around the AT-rich island to which it maps (see Table SI in the supplemental material; also see Materials and Methods). We identified the origins utilized in the first endoreduplication cycle of

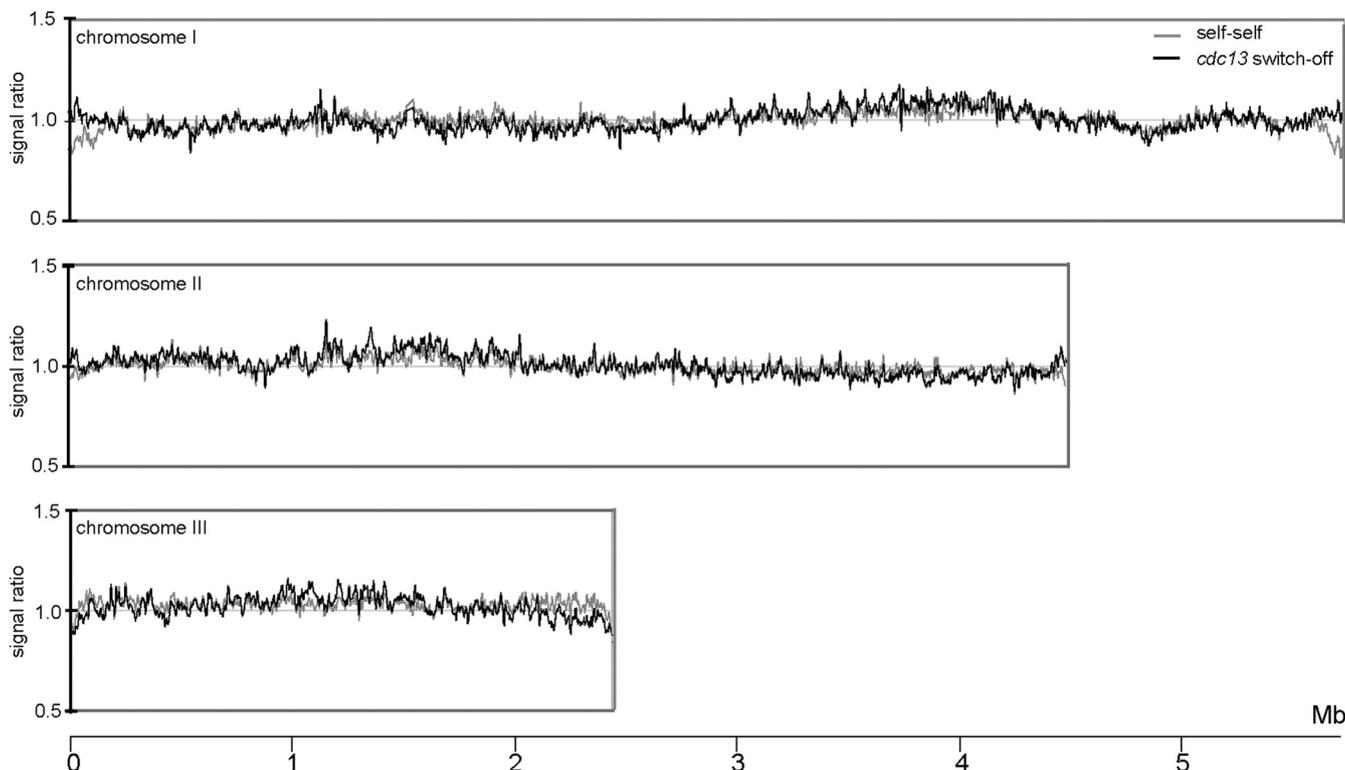


FIG. 4. Equal replication across the genome in a *cdc13* switch-off strain. DNA from rereplicating cells which had attained 16C and 32C DNA contents in the absence of HU was hybridized against a reference to microarrays. Signal was normalized to the rest of the genome.

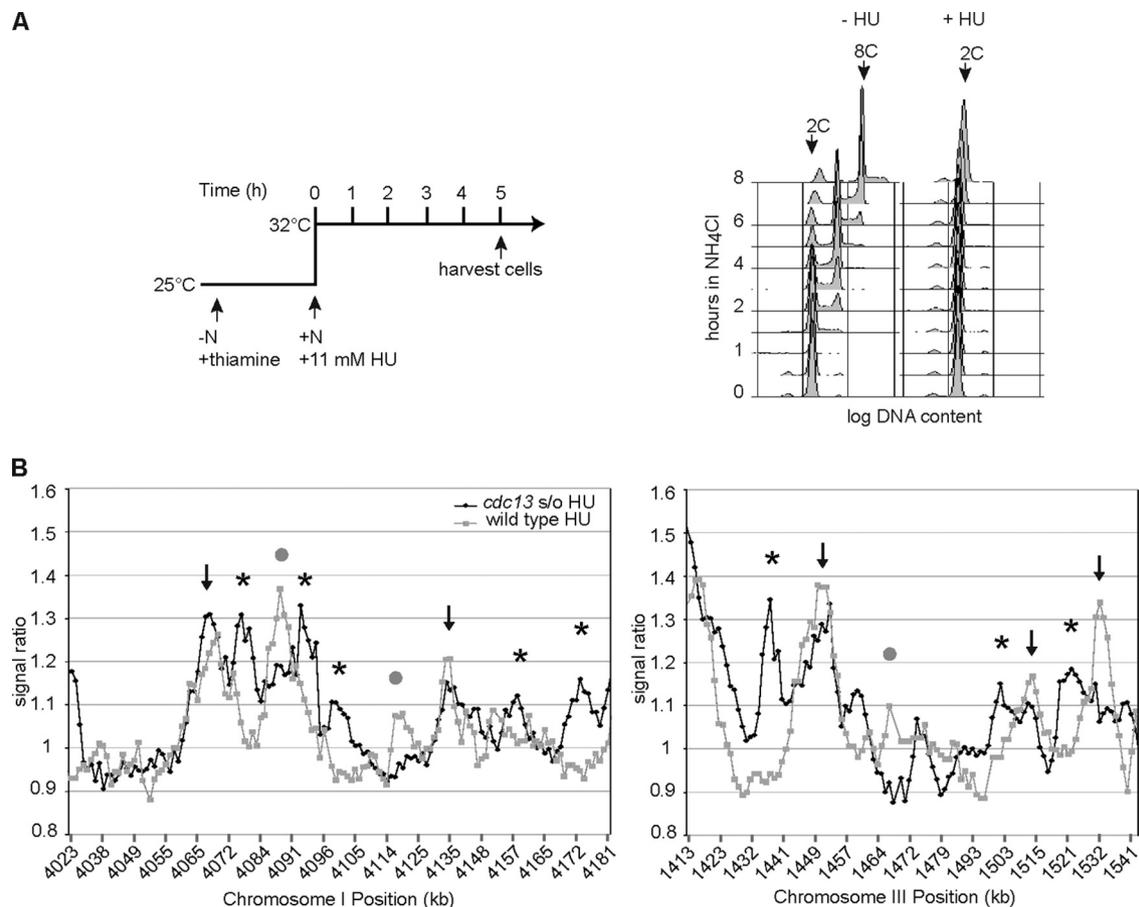


FIG. 5. Replication origin usage in a *cdc13* cyclin switch-off. Mapping origins in endoreduplication of a *cdc13* cyclin switch-off mutant. (A) Left panel: schematic of the *cdc13* cyclin switch-off time course experiment. Cells were nitrogen starved for 5 h at 25°C in the presence of thiamine. Nitrogen was replenished and the culture shifted to 32°C, and 11 mM HU was added. Right panel: FACS analysis of the *cdc13* switch-off mutant, induced into endoreduplication with or without the addition of 11 mM HU. In the presence of HU, cells block at the onset of the first round of endoreduplication. In the absence of HU, cells undergo repeated rounds of replication without intervening mitoses. (B) Comparison of wild-type and *cdc13* switch-off replication profiles from the average for two HU experiments. Examples of origins: arrows mark origins which are used both in a normal S phase and in the *cdc13* s/o strain, gray circles mark normal S-phase origins not used in *cdc13* s/o, and asterisks mark new origins which are used only in the *cdc13* s/o strain. The regions were selected for illustration because they contain all three types of origins.

*cdc13* s/o using microarray analyses of cells treated with 11 mM HU. Samples were taken at 5 h (Fig. 5A), when most cells of the culture not treated with HU had undergone a doubling in DNA content. Examples of the microarray profiles are given in Fig. 5B for two regions of the genome, where peaks in the signal ratio can be observed which mark the locations of fired origins. These data are compared to data from wild-type cells undergoing replication in a normal cell cycle to determine if the same origins are used.

A total of 799 origins were identified, a number roughly similar to the 904 identified in a normal S phase (Table 2; see Table SI in the supplemental material). Of these 799 origins, 697 are active in a normal S phase and 102 are not used in a normal S phase, while 207 normal S-phase origins are not fired in *cdc13* s/o (Table 3). The percentage of AT content (see Materials and Methods) and the intergenic length, both of which are known to be correlated with origin strength (9), did not differ on average among these groups (Tables 2 and 3). The mean AT contents were similar, between 72.1% and 74.0% (intergenic regions in the genome contain ~70% AT on aver-

age [9, 38]), and the mean AT content for all origins activated in *cdc13* s/o was identical to that in S phase (73.8%) (Table 2). The lengths of the intergenic regions in which the origins are embedded were also similar in inactive and active mitotic origins, with average lengths ranging from 1,349 to 1,776 bp (average intergenic distance in the genome overall is 960 bp). As previously described, AT-rich sequences acting as replication origins are preferentially located in intergenic regions of divergent transcription (38), which are longer than the average

TABLE 2. Origins used in mitotic S phase and in endoreduplication of *cdc13* s/o<sup>a</sup>

Replication program	No. of origins	Mean AT content (%)	Mean intergenic distance (bp)	Mean mitosis efficiency (%)
Mitotic S phase	904	73.8	1,678	17.6
<i>cdc13</i> s/o	799	73.8	1,737	18.4 <sup>b</sup>

<sup>a</sup> For definitions of all parameters, see Materials and Methods.

<sup>b</sup> Not including the additional origins which do not fire in the mitotic cell cycle.

TABLE 3. Categories of origins according to usage in endoreduplication<sup>a</sup>

Category	No. of origins in category	Mean AT content (%)	Mean intergenic distance (bp)	Mean mitosis efficiency (%)	Mean <i>cdc13</i> s/o efficiency (%)
Mitotic origins used in <i>cdc13</i> s/o	697	74.0	1,776	18.4	13.8
Nonmitotic origins used in <i>cdc13</i> s/o	102	72.1	1,470	NA <sup>b</sup>	18.1
Mitotic origins not used in <i>cdc13</i> s/o	207	73.2	1,349	14.9	NA <sup>c</sup>
Genome avg		~70 <sup>d</sup>	960 <sup>e</sup>		

<sup>a</sup> Parameters are defined in Materials and Methods.

<sup>b</sup> These origins do not fire in mitotic S phase, so there is no mitosis origin efficiency.

<sup>c</sup> These origins do not fire in *cdc13* s/o, so there is no *cdc13* s/o origin efficiency.

<sup>d</sup> Refers to all intergenes, genome-wide (9, 38).

<sup>e</sup> Refers to all intergenic regions in the genome, regardless of origin activity.

*S. pombe* intergene region (42), and a similar bias was reflected in the wild-type and *cdc13* s/o origins. We also found that there was no correlation of activated or suppressed origins with loci of cell cycle-regulated genes. Although many of the same origins were used, their efficiency in *cdc13* s/o was not well correlated to wild-type efficiency ( $r^2$  value of 0.3). We conclude that origins used in *cdc13* s/o are mostly similar to those of a normal S phase, although with changes in efficiency.

We examined whether the new origins activated in *cdc13* s/o were sites on the chromosome where pre-RCs assemble. The new origins mapped were compared with the previously reported genome-wide localization of Orc4, a component of the pre-RC, in G<sub>1</sub> (16) (see Table SI in the supplemental material). We devised a script to search the data set of Hayashi et al. for Orc4 localization in the vicinity of our origins using two signal ratio thresholds (see Materials and Methods). With the lower threshold, 96% of the 401 strong origins reported in the work of Heichinger et al. (18) had Orc4 localization, and 69% of the 503 weaker origins reported here were colocalized with Orc4. These values should be compared with the 74% of the 102 *cdc13* s/o-activated origins which were colocalized with Orc4. Applying a higher threshold similar to that of Hayashi et al. (16) (see Materials and Methods), 92% of the strong wild-type origins colocalized with Orc4 while 50% of the activated *cdc13* s/o origins and 50% of weaker wild-type origins showed Orc4 binding. We conclude that the extent of Orc4 binding (reflecting pre-RC formation) to the new origins activated in *cdc13* s/o was similar to the weaker normal S-phase origins but less than the stronger normal S-phase origins.

## DISCUSSION

We have shown that in the absence of G<sub>2</sub>/M CDK, DNA synthesis and the accompanying G<sub>1</sub>/S gene expression are periodic and are coordinated with cell volume increases at approximate multiples of the minimum cell size for the G<sub>1</sub>/S transition. Most of the origins used are also utilized in a normal mitotic S phase, although some cryptic origins are activated mostly at sites of normal pre-RC formation and there are changes in origin efficiency compared with a normal S phase. Finally, we have shown that there is essentially equal replication across the genome.

Given these results, we propose that each period of DNA synthesis corresponds to a complete round of DNA replication as observed in a mitotic cell cycle. In the normal cell cycle, CDK activity decreases as cells exit mitosis due to destruction

of Cdc13, sending cells into G<sub>1</sub> of a new cycle. Here, by switching off *cdc13* in G<sub>2</sub>, G<sub>2</sub>/M CDK activity is decreased and cells are unable to undergo mitosis while inhibition of S phase is lost. Rather than undergo aberrant unprogrammed DNA synthesis, cells progress through an abbreviated cycle, returning to a G<sub>1</sub>-like state. They then reenter a normal G<sub>1</sub>/S program and carry out S phase. Perturbing the level of CDK activity resets the cell cycle into endoreduplication, emphasizing CDK as a major cell cycle regulator important for maintenance of ploidy. What could drive periodic G<sub>1</sub>/S gene expression and S phases in the absence of a normal cell cycle? The MBF-dependent cyclin Cig2 is one candidate, because together with Cdc2 it phosphorylates Res1 to inhibit MBF, constituting an autoregulatory negative feedback loop (2). Cig2 oscillation seems to be necessary for periodic endoreduplication in *cdc13* s/o and might drive oscillation of other MBF-dependent genes required for S phase (14, 23, 29, 43).

*Cdc13* s/o cells replicate the genome in complete rounds, maintaining the characteristic that each part of the genome is replicated only once per S phase. Nearly all origins used to replicate the genome in the first reduplication cycle are S-phase origins (87%), and the total number of origins is roughly similar at 799, compared to 904 in wild-type S phase. Mitotic S-phase origins were used at a slightly lower mean efficiency in endoreduplication than in a normal S phase (~13.8% versus ~18.4%), while the newly activated origins were used at 18.1% efficiency. These results are consistent with the view that there is a limited potential to activate a subset of potential origins or assembled pre-RCs in S phase (9, 16, 24, 33). The program of origin usage may differ when cells are replicating from G<sub>2</sub>, perhaps because gene products normally present in S phase are absent or because chromatin has not been condensed by an intervening M phase subsequent to the previous S phase. We have found that during a normal S phase, Orc4 localizes to a significant number of the origins that are newly activated in the *cdc13* s/o strain, suggesting that pre-RCs form at many of the sites which can be activated during endoreduplication. Finally, we have shown that although the efficiencies of individual origins differ, the net outcome is even replication across the genome.

Similarly to fission yeast, in endocycles of *Drosophila melanogaster* ovarian follicle cells, BrdU incorporation is periodic, nearly the whole genome is replicated per round of DNA synthesis, and the increase in ploidy is correlated with a cell volume increase (6, 12, 25). The transition from mitosis to

endocycling is brought about by inhibition of M-phase-promoting CDK activity (39), and periodic rounds of DNA synthesis in follicle cells rely on fluctuation of the G<sub>1</sub>/S cyclin-CDK complex (reviewed in reference 7). We conclude that more generally in eukaryotes, G<sub>2</sub>/M CDK activity restricts S phase to once per cell cycle, and its absence leads to endoreduplication with repeated S phases in the absence of mitosis.

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